METABOLISM OF THE OESTROGENS AND THEIR CONJUGATES

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STATEMENT AS TO AUTHOR'S PARTICIPATION

The publications and other data in this D.Sc. thesis were the result of my own participation. In more than half of the work I contributed directly in that I was personally involved in performing the experiments, with or without technical assistance. In the greater part of the remainder of the work I supervised, closely and directly, the laboratory activities of graduate students in the M.Sc. and/or Ph.D. courses. In the case of certain M.Sc. candidates additional work to that described in their theses was carried out by me and my technical staff prior to publication in a journal. In some instances collaboration with outside investigators (notably Professor D.S. Layne) resulted in publications. In these cases at least half of the work, and in most instances much more, was carried out in my own laboratory by me or under my direct supervision. All of the *in vivo* studies involving human subjects were performed ethically with the collaboration of a clinician (mainly Dr. P.R. Blahey).

Signature

Signed at London, Ontario, Canada

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ABSTRACT

Optimal conditions for hydrolysis of urinary oestrogen conjugates in the human have been sought. A β-glucuronidase-sulphatase mixture (H. plmatia) is shown to be effective in cleaving the sulphate and glucosiduronate forms of some eight metabolites. A bacterial (E. coli) β-glucuronidase preparation is particularly active in hydrolysing both oestrogen and 17-ketosteroid glucosiduronates. Using these techniques and other improved methodology, levels of seven or eight oestrogen metabolites have been measured in normal and diabetic pregnancy (human). Apparent abnormalities are seen in the diabetic state and tentative conclusions are drawn regarding sites of production of certain oestrogens during pregnancy.

Metabolism of some free oestrogens have been studied in the human *in vivo* and *in vitro* and in certain other species *in vitro*. Oestriol and 16-epioestriol are shown to be not necessarily 'end products' of metabolism but to be variably metabolized by laying hen liver, erythrocytes of certain species and by the human *in vivo*. The 2-methoxylation of oestrone has been studied in rat liver fractions and the metabolism of the clinically useful synthetic compound 17α-ethynyloestradiol-17β-3-cyclopentyl ether has been delineated in the human.

The conjugation patterns of urinary oestrogen metabolites in the pregnant and non-pregnant human are shown to be similar by a differential hydrolytic technique. The quantitative importance of oestrone in the sulphate fraction has been established as well as that of 16α-hydroxyoestrone and 16-ketooestradiol-17β in the

sulphoglucosiduronate fraction.

It has been shown that tissues from the laying hen are more active, with respect to sulphurylation, towards phenolic steroids than to dehydroisoandrosterone. Also, liver from the same species directly interconverts the 3-sulphates of oestrone and 17β-oestradiol. The ability of a rat liver preparation to sulphurylate the 3 position of phenolic steroids appears to be related to the constituents of ring D of the steroid.

The glucosiduronates and sulphates of oestrone and 17β-oestradiol have been successfully separated chromatographically and the procedure has been employed to prove that the human conjugates 17β-oestradiol to yield both its 3- and 17-monoglucosiduronates. The fates of the latter two conjugates differ *in vivo* in that the former is subjected mainly to 17-dehydrogenation and little deconjugation, whereas the latter undergoes an enterohepatic type of metabolism with considerable deconjugation and reconjugation. The metabolism of the monoglucosides of 17β-oestradiol in the human has been studied. The 3-glucoside residue is rapidly removed with the release, and extensive metabolism, of 17β-oestradiol. The 17-glucoside residue is much less readily removed and the metabolism of the 17-glucosides of 17β-oestradiol and 17α-oestradiol shows some resemblance to that of 17β-oestradiol-17-glucosiduronate.

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GENERAL INTRODUCTION

This thesis incorporates studies performed over the approximate period 1958-1971. In the late fifties and early sixties, due largely to the isolation of many new oestrogen metabolites by Marrian et al. (1-4) and others (5-8), one of the main questions concerned the manner in which so many metabolites arose from the secreted hormone (now considered to be mainly 17β -oestradiol*) in the human. This necessitated several lines of study, involving (a) methodology (including means of conjugate hydrolysis) for the measurement of oestrogen metabolites other than oestrone, oestradiol and oestriol, the latter having been so ably pioneered by Brown (9) and Bauld (10); (b) the mode of interconversion and formation of the newer metabolites in their free, unconjugated form; (c) methodology for the direct separation of steroid conjugates so as not to rely too heavily on direct hydrolysis of crude mixtures of these compounds as a means of identification; (d) the possibility of metabolism of oestrogen conjugates such as glucosiduronates and sulphates.

The greater part of the work here submitted involves various aspects of methodology and metabolism, almost wholly of the oestrogens, as they concern the human subject. In addition, however, studies on certain small animal species are included. These latter, mostly performed in vitro, have been particularly

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*Hereafter referred to as oestradiol except where distinction from 17α -oestradiol is necessary.

helpful in the biosynthesis of certain conjugated steroids required for human studies.

In the author's laboratory, studies on conditions for enzymatic and acid hydrolysis of steroid conjugates, along with other methodology (Section A), produced for the first time a quantitatively meaningful pattern of several oestrogens besides oestrone, oestradiol and oestriol in human pregnancy urine (Section B). Besides this, patterns of oestrogens in diabetic pregnancy urine were established (Section B). These above data were, however, obtained without regard to the specific type of conjugation of the urinary steroids and did not, therefore, elucidate the conjugation pattern.

In addition to the above, considerable information was obtained, both in vivo and in vitro with human and other species, regarding the interrelationships of various phenolic steroids, particularly the 16-oxygenated compounds, in their unconjugated forms (Section C).

Further studies, involving more or less specific means of hydrolysis, e.g., β-glucuronidase incubation and solvolysis, to split glucosiduronates and sulphates, respectively, shed some light, mostly indirect, on patterns of oestrogen conjugate metabolites in both pregnant and non-pregnant individuals (Section D). This study also resulted in establishing without question that oestrone-3-sulphate can be quantitatively, in some humans, a most important urinary metabolite.

The formation and metabolism of oestrogen sulphates, *in vitro* and *in vivo*, has been explored to some degree in species other than the human (Section E). Particularly useful information has been obtained in this respect using as a test species the laying hen (Gallus domesticus) since conjugation of oestrogens in this species *in vivo* appears to be confined to sulphurylation (10a).

The necessity for methodology which would more directly allow of the study of steroid conjugate patterns and metabolism, e.g., the direct separation of intact conjugates, became obvious. The original studies of Hahnel (11,12) utilizing DEAE-Sephadex chromatography to achieve this in a partial fashion, presented a basic procedure which was modified in the author's laboratory to successfully separate several conjugates of oestrone and oestradiol (Section F). This technique was then employed to describe, for the first time, the interconversions and biosynthesis of various conjugates, notably the glucosiduronates of oestrone and oestradiol in the human subject (Section G). Information was then obtained for the metabolism of the 3- and 17-glucoside conjugates of 17β-oestradiol and the 17-glucoside of 17α-oestradiol in the human *in vivo* (Section H). This latter was performed in order to compare the behaviour of charged and relatively uncharged conjugates of otherwise very similar structure.

SECTION A

ASPECTS OF CONJUGATE HYDROLYSIS AND STEROID METHODOLOGY

INTRODUCTION

A great deal of work in the last decade has centred around the measurement of oestrogens, particularly oestriol, in diabetic, and other compromised pregnancies, in view of the high intrauterine death rate and the possibility that oestrogens may be usefully monitored with a view to more effective control and treatment of pregnancy (13-19). A necessary step in all earlier, and many current methods, was hot acid hydrolysis to release the steroids from conjugation. Brown and Blair (20), in an attempt to establish optimum conditions for this step, mentioned an apparent destructive effect of glucose, in the presence of hot acid, on the oestrogens. These same studies established conditions for the enzymatic hydrolysis of urinary oestrone, oestradiol and oestriol conjugates using a preparation from the common limpet (Patella vulgata) which contained both β-glucuronidase and phenolsulphatase(s). These early data, together with the isolation of many new oestrogen metabolites (1-8) necessitated enquiry into suitable procedures for hydrolyzing the conjugated forms of these (besides those of the three 'classical' oestrogens and of the urinary 17-ketosteroids) prior to successfully measuring the steroids released.

The methods of Brown (9) and Bauld (10) were the first capable of measuring with some reliability the levels of the three 'classical' oestrogens, oestrone, oestradiol and oestriol,

in the urine of the non-pregnant human. Unfortunately, these techniques came to be frequently used in situations where their sensitivities were not adequate. Thus data were seen which fell below the useful working range of the procedures. Also, the method of Bauld appeared to yield certain values which were higher than expected (21) and it therefore became necessary to evaluate this latter procedure in several modified forms.

Discussion of Published Work (Appendix - Section A)

The apparent destructive effect of glucose on classical oestrogen conjugates during hot acid hydrolysis, and the manner in which this can be overcome, either by prior high dilution of the urine or by enzymatic means, has been published (Hobkirk, Alfheim and Bugge, J Clin Endocr 19: 1352, 1959). This information has proved most useful to the author and others in the measurement of urinary oestrogens in pregnancy (19, 22-24).

A comparison of the relative abilities of several enzyme preparations to hydrolyze the conjugated forms of some six urinary oestrogen metabolites in pregnancy urine led to the establishment of suitable conditions for incubation (Bugge, Nilsen, Metcalfe-Gibson and Hobkirk, Can J Biochem Physiol 39: 1501, 1961). The enzyme preparations employed were; a highly purified beef liver β-glucuronidase (Ketodase), a bacterial β-glucuronidase preparation (E. coli), a crude mixture of enzymes, including β-glucuronidase and sulphatase(s), from the edible snail (Helix pomatia), and a similar mixture from the common limpet. The snail preparation appeared to be the one of

choice where overall hydrolysis of urinary oestrogen conjugates was desired. The bacterial preparation was most efficient for glucosiduronate hydrolysis. Thus for the first time conditions were established for the optimum hydrolysis of oestrogen conjugates other than those of the classical three. Particularly important in this respect was the successful hydrolysis of the conjugates of the rather unstable 16α-hydroxyoestrone and 16-ketooestradiol-17β* with apparently minimal destruction. This information has been used by other investigators (24-26), and by the author (Hobkirk and Nilsen, J Clin Endocr 22: pp 134 and 142, 1962; see Section B) in the elucidation of the pattern of some seven steroids in pregnancy urine which at the time of study was not at all known.

A somewhat similar enzymatic study was performed with respect to urinary 17-ketosteroid conjugates in the human (Hobkirk and Cohen, Can J Biochem Physiol 38: 769, 1960). An interesting stereochemical effect was apparent. Over the incubation periods studied (12-96 hr) the 5β steroids (aetiocholane series, including aetiocholanolone and its 11β-hydroxy and 11-keto forms) were released from conjugation relatively readily and to similar extents by the four enzyme preparations. The 5α steroids (androstane series, including androsterone and its 11β-hydroxy form) on the other hand, were released efficiently by the bacterial preparation but less so by the other enzymes,

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*Referred to hereafter as 16-ketooestradiol.

notably the beef liver β-glucuronidase. Indeed, the latter enzyme preparation, at concentrations which released maximum amounts of the 5β steroids, released only a fraction of the 5α steroids liberated by the bacterial preparation. However, at very high concentrations of the liver enzyme liberation of androsterone approached that seen with the bacterial preparation. It seemed likely, therefore, that hydrolysis with the liver preparation could lead to very erroneous results for the urinary levels of the 5α steroids and might well suggest the presence of 'unknown' conjugates rather than indicate a structural effect (see reference 27). These data demonstrate the need for care in the choice of enzymes and in the interpretation of results. They have been adequately borne out by Beale et al. in studies reported in 1969 (27a).

The requirement for increased sensitivity and specificity in the measurement of oestrone, oestradiol and oestriol by Bauld's method led to an investigation of a modified technique (Hobkirk and Nilsen, Steroids 3: 453, 1964). The incorporation of the Ittrich fluorimetric procedure unquestionably influenced results for the better and a combined procedure adapted from the methods of Bauld (10) and Givner et al. (28) produced data for males and post-menopausal females which compared well with those of other authors using mainly the Brown technique or modifications of it. Spectrophotometry and fluorimetry yielded similar values in the modified method. The study resulted in a good technique for later use in the author's laboratory.

Attempts to accurately quantitate ring D α-ketol phenolic steroids at this stage yielded results of doubtful validity.

Unpublished Data Related to the Above*

It appears that a bacterial β-glucuronidase preparation is exceptionally active in hydrolyzing both urinary oestrogen and 17-ketosteroid conjugates (presumably glucosiduronates). The data in Table 1 (obtained by the method of Bauld) show that as little as 2 units of activity per ml of urine were as active as 20 units per ml in liberating the three classical oestrogens from conjugation in non-pregnancy urine within 24 hours. The bacterial preparation gave rather similar results to, although somewhat better than, those obtained by hot acid, liver enzyme and limpet enzyme hydrolysis. It is to be expected, however, that in urines containing considerable oestrogen sulphates (see Section B) the molluscan preparations would be of great value. The lower results obtained with the snail preparation may have been due to particularly troublesome emulsions formed on extraction at the high enzyme-protein concentrations employed. The amounts of enzymes used were based on optimal or excess requirements reported by other investigators. The reason for the particularly high activity of the bacterial preparation was sought.

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*This material has actually appeared in book form as part of the proceedings of a United States N.I.H. Workshop on Estrogen Methodology held in 1963.

Reference: Estrogen Assays in Clinical Medicine, ed. C. A. Paulsen, Univ. of Washington Press, 1965, pp. 10-18.

TABLE 1

Measurement of Oestrone, Oestradiol and Oestriol after Hydrolysis by Various Methods in Pooled Luteal Phase Urine from 5 Women without Medication.

Hydrolytic conditions*	Oestrogen Levels (ug per 1/5 urine pool) Oestriol	Oestrone	Oestradiol	Total
Boiling with 15 vol % HCl, 1 hr	21.0	8.0	4.9	33.9
2 Units bacterial enzyme/ ml, pH 6.5, 24 hr	23.3	8.4	4.8	36.5
20 Units bacterial enzyme as above	21.9	10.2	4.0	36.1
300 Units liver enzyme/ ml, pH 5.0, 120 hr	20.1	8.5	4.2	32.8
1000 Units limpet enzyme/ ml, pH 4.6, 96 hr	20.5	7.6	3.9	32.0
1500 Units snail enzyme/ ml, pH 5.2, 24 hr	15.0	7.1	3.7	25.8

*Incubations were at 37°C. The unit of activity is based on the ability of each preparation to hydrolyze phenolphthalein glucuronide at the respective pH values shown; i.e., it approximates a Fishman unit and is, therefore, only a measure of β-glucuronidase content.

Table 2 shows relative activities of the various enzyme preparations towards oestriol glucosiduronate (isolated from human pregnancy urine and presumably mainly oestriol-16-glucosiduronate) as substrate. Although the bacterial preparation displays maximum activity near neutral pH, relatively small amounts (cf. other preparations) were still active at pH 5.0. Also, the inhibitory effect of urine was much more marked at pH 5.0 than at 6.5 for the bacterial enzyme and it was very high for the other preparations at pH 4.5 - 5.2. In addition it was noted (Fig. 1) that inhibition of the bacterial β-glucuronidase with boiled saccharate solutions was extremely difficult at pH 6.5 compared with the picture for the other enzymes at their best pH values. This was also true of the endogenous inhibitors present in urine (Fig. 1). Even at pH 5.0 inhibition of the bacterial enzyme by boiled saccharate was less than for the other enzymes at pH 4.5 - 5.2, although the presence of urine caused marked lowering of the activity of enzymes from all sources at around pH 5. Supporting data are shown for phenolphthalein glucuronide as substrate in Table 3. For all enzyme preparations much larger concentrations of inhibitor were required at pH 6.5 than at 4.5 - 5.2 to yield comparable inhibition. This is presumably due to the greater stability of the active inhibitor, saccharo-1, 4-lactone at the lower pH values (29). Endogenous urinary inhibitors of this type would probably behave similarly during enzymatic hydrolysis. Thus the value of bacterial β-glucuronidase may reside partly in the

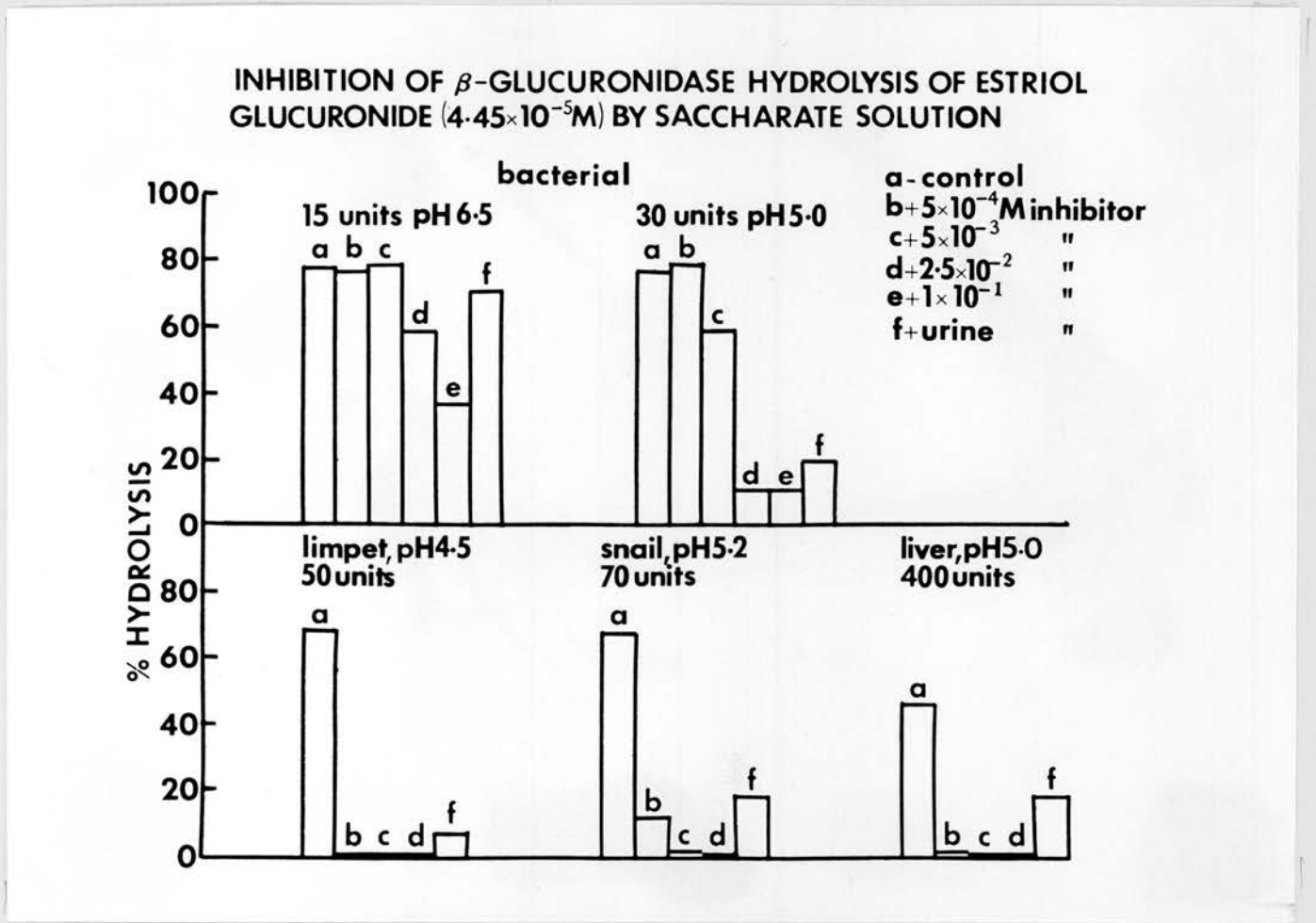
INHIBITION OF β-GLUCURONIDASE HYDROLYSIS OF ESTRIOL GLUCURONIDE (4·45×10⁻⁵M) BY SACCHARATE SOLUTION
bacterial
a-control
b+5×10⁻⁴M inhibitor
c+5×10⁻³ "
d+2·5×10⁻² "
e+1×10⁻¹ "
f+urine "
15 units pH 6·5
30 units pH 5·0
limpet, pH 4·5
50 units
snail, pH 5·2
70 units
liver, pH 5·0
400 units
% HYDROLYSIS
100
80
60
40
20
0

Figure 1

TABLE 2

Approximate Amounts of β-Glucuronidase Preparations Required to Yield Equal Degrees of Hydrolysis of Oestriol Glucosiduronate (4.45×10^{-5}M) in 16 hr at 37°C.

Enzyme preparation	pH	Units of β-Glucuronidase per ml	
		Pure Substrate	Substrate + Urine *#
Bacterial	6.5	15	17, 19
Bacterial	5.0	30	120, 50
Liver	5.0	600-700	1600-1700
Limpet	4.5	50-60	400-500
Snail	5.2	80-90	250-350

* Urine (0.5 ml) accounted for one-half of total reaction volume.

Two urines were used for the bacterial preparation; one for the others.

TABLE 3

Effect of pH on the Inhibition of β-Glucuronidase Activity by Boiled Saccharate Solutions (Phenolphthalein Glucuronide used as Substrate)

Enzyme* preparation	Molarity of Substrate	pH	Molarity of Saccharate giving 50% inhibition
Bacterial	2×10^{-4}	5.0	5.2×10^{-2}
"	"	6.5	1×10^{-1}
Liver	1×10^{-3}	5.0	3.6×10^{-4}
"	"	6.5	5.2×10^{-2}
Limpet	1×10^{-3}	4.5	1×10^{-4}
"	"	6.5	1.3×10^{-2}
Snail	1×10^{-3}	5.2	1.9×10^{-3}
	"	6.5	1×10^{-1}

* Fifty units of β-glucuronidase activity were used in all incubations at each pH value.

position of its optimum pH and partly in its 'basic resistance' to inhibition. Moreover, its apparently low specificity towards the stereochemistry of the A/B ring fusion in its substrates (Hobkirk and Cohen, Can J Biochem Physiol 38: 769, 1960) further commends it as a means of hydrolyzing steroid glucosiduronates. It should be noted that Jayle et al. in 1959 (30) had claimed that the snail enzyme preparation was better than bacterial β-glucuronidase or hot acid hydrolysis in releasing urinary oestrone, oestradiol and oestriol from conjugation. In 1962 Kirk et al. (25) showed that the bacterial preparation was superior to the mammalian liver β-glucuronidase for release of 17-ketosteroids and oestrogens.

Recent work by Roy and Slaunwhite (31), and also unpublished observations in the author's laboratory, showed that a steroid 17β-hydroxy dehydrogenase could be present in some batches of bacterial β-glucuronidase. In none of the studies reported in this thesis was such material utilized. In later work in which oestrone and oestradiol conjugates were purified in the intact form (see Section G) the highly purified liver β-glucuronidase was generally employed in hydrolysis. It should be noted that claims have been made (32, 32a) that certain β-glucuronidase-containing preparations, including that of bacterial origin, may cause some alteration in the structure of the steroids hydrolyzed.

SECTION B

URINARY OESTROGEN LEVELS IN PREGNANCY WITHOUT REGARD TO SPECIFIC MODES OF CONJUGATION

INTRODUCTION

Although urinary oestriol in pregnancy had been measurable for some time it was not until the advent of the Brown (9) and Bauld (10) methods that it and its companion compounds, oestrone and oestradiol, could be quantitated with some certainty. Brown established the pattern of these steroids in normal pregnancy in 1956 (33) but left unresolved the picture for diabetic pregnancy, a condition associated with a high intrauterine death rate. The isolation of many more oestrogen metabolites (1-7, 34) required that the pattern of at least some of these be established as well. The availability of information on the hydrolysis of the conjugates of several of these oestrogens (see Section A, above) was the first step towards an understanding of the problem, and a modification of the procedure of Givner et al. (28), made in the author's laboratory, allowed the measurement of the three classical oestrogens together with 2-methoxyoestrone, 16-epioestriol and the ring D α -ketols (mainly 16α-hydroxyoestrone and 16-ketooestradiol) to be made. These studies were designed to yield information on overall patterns without specific relation to conjugation but, hopefully, by the use of methods where optimal hydrolysis was achieved.

Discussion of Published Work (Appendix - Section B)

By laboratory procedures which were reliable, data were obtained which suggested that, although oestrone and oestradiol levels were within normal range in most diabetic pregnancies, oestriol was, on the average, low (Hobkirk, Blahey, Alfheim, Raeside and Joron, J Clin Endocr 20: 805, 1960). With the exception of one early publication (35) dealing with a very few subjects, this appears to be the first published study, using modern techniques, on urinary oestrogens in diabetic pregnancy. The above-mentioned work of Rubin et al. (35) concluded that oestrogens may be low in diabetic subjects with pre-eclamptic toxemia but normal in diabetes without associated toxemia. It became known over the period 1962-63, after much study (14, 15, 18, 19), that urinary oestriol levels may be closely related to foetal condition and it is likely, indeed probable, that, in our original paper, lowest oestriol levels were associated with foetal distress. More specifically clinical aspects of such studies were published by Joron, Hobkirk, Blahey, Routledge and Fowler (Diabetes II, 514, 1962) which showed that the behaviour of urinary oestriol was indicative of foetal viability.

In 1962 two publications (Hobkirk and Nilsen, J Clin Endocr 22: pp. 134 and 142, 1962) established the pattern of several of the newer oestrogen metabolites, together with the classical compounds, in both normal and diabetic pregnancy. The normal values were to prove remarkably similar to those

quoted by Breuer in 1965 (36), using mainly paper chromatography, although the latter investigator did not show data for the likely variation between individuals. He did, however, give data for a large number of metabolites besides those measured by this author. Others who have reported procedures for measurement of urinary oestrogens in addition to the classical three are Wotiz and Chattoraj in 1964 (37), and Eechaute et al. in 1967 (38), both of whom reported values similar to the author's although their data were more limited in number. The former investigators (37) utilized gas-liquid chromatography, the latter (38), silica gel chromatography and fluorimetry for analysis. The most recent data available have been published by Adlercreutz and Luukkainen in 1970 (39) in which a large number of oestrogens and metabolites were measured, and identified, in various biological fluids in pregnancy by gas-liquid chromatography combined with mass spectrometry. The urinary values are quite comparable with those of eight years standing published by the present author. In the papers of Hobkirk et al. it was noted that the ring D α-ketols were quantitatively important metabolites and appeared to be higher in diabetic than in normal pregnancy. Also, in some individuals urinary oestrone was markedly elevated in diabetic pregnancy (see also Hobkirk et al., J Clin Endocr 20: 805, 1960). In addition, 2-methoxyoestrone appeared to show a trend in excretion during the second half of pregnancy which was different from the other metabolites and was frequently,

apparently, absent even in late pregnancy. This point was studied in more depth using a modified, and more sensitive procedure (Hobkirk and Nilsen, J Clin Endocr 23: 274, 1963). It was confirmed that 2-methoxyoestrone frequently did not behave as did total oestrogens during the last 25 weeks, or so, of pregnancy, being more or less immeasurable in some and relatively high (up to 1 mg/24 hrs, corrected for losses) in others. Wotiz and Chattoraj in 1964 (37) and Adlercreutz and Luukkainen in 1970 (39) have arrived at substantially the same conclusion. It was suggested in the present author's publication, based on limited data, that the behaviour of 2-methoxyoestrone might be due to a variable degree of demethylation in vivo as shown for the 3-methyl ether of oestrogens by Brown (40) and, as published in 1964, for the 2-methoxy group, under different experimental conditions, by Breuer et al. (41). More recent work by Yoshizawa and Fishman (42), using radioactively labelled 2-methoxyoestrone (entirely unavailable to the present author in the early sixties) strongly suggests that no demethylation occurs. There can, however, be little question of the data for endogenous 2-methoxyoestrone patterns although no further explanation appears to have been forwarded as to the shape of the excretion curves.

A further publication by Hobkirk, Anuman-Rajadhon, Nilsen and Blahey (Clin Chem 16: 235, 1969) has confirmed the elevation of ring D α-ketols in diabetic pregnancy mentioned above. One wonders whether this is due to a difference in

peripheral conversion of oestrogens and their metabolites in the tissues of the diabetic mother or to foetal production. It certainly may be of some interest with respect to the measurement of oestriol alone or 'total oestrogens' in the maternal urine with a view to monitoring the risk of foetal loss. Along these lines it should be noted that S. L. Cohen has recently claimed (43, 44) through somewhat indirect means, that in diabetic pregnancy a group of chemically labile oestrogens (including perhaps 2- and 6-hydroxylated compounds) is vastly increased as compared with normal pregnancy. As far as the present author is aware, no study has been reported on oestrogen metabolism in non-pregnant diabetic humans.

Measurement of the specific radioactivities of seven urinary metabolites of intravenously administered oestradiol-6, 7-^{3}H in the pregnant woman showed that the values for the individual ring D α-ketols (16α-hydroxyoestrone and 16-ketooestradiol) were some twofold those of the 16-desoxy oestrogens but were in turn considerably (four to seven times) higher than that of oestriol (Hobkirk and Nilsen, J Clin Endocr 26: 625, 1966). This suggested that a urinary metabolite such as 16-ketooestradiol was probably not, for the main part, a conversion product of the foetal oestriol pool since, if it were, there would presumably have been a marked lowering of specific radioactivity to a value somewhat similar to that for urinary oestriol. Following publication of the above study Diczfalusy (45) showed that 16α-hydroxyoestrone, a major

foetal steroid, is largely converted to oestriol in the placenta and forms a major source of urinary oestriol in pregnancy. Also, Reynolds et al. in 1968 (46) have suggested that urinary 16 α-hydroxyoestrone in particular may be largely a maternal metabolite in pregnancy. All of this information raises the question of the origin of 16-oxygenated oestrogens other than oestriol in pregnancy and points out the necessity for an understanding of this in the measurement of urinary oestriol or 'total oestrogens', particularly in diabetic pregnancy, as a means of studying foetal viability.

Results obtained by Siiteri and MacDonald in 1966 (47) and by Adlercreutz and Luukkainen in 1970 (39) show that about 50% of the oestradiol produced in pregnancy arises in the maternal compartment whereas only some 10% of oestriol is so produced. This information, together with the specific radioactivities of the various urinary oestrogens published by the author (Hobkirk and Nilsen, J Clin Endocr 26: 625, 1966) would indicate that a much greater fraction of the ring D α-ketols and 16-epioestriol than of oestriol, is of maternal origin. The specific activities alone might indicate that the above maternal fraction for the ring D α-ketols and 16-epioestriol should be closer to that for oestradiol, i.e., 50%, than for oestriol, i.e., 10%. However, this assumes some linearity between urinary specific activity and dilution with endogenous metabolites of various origins and may not be warranted in view of the complexity of the prevailing circumstances. The most definitive data now in

existence (39), in which these steroids were measured by the newest techniques in maternal urine and plasma and in cord plasma and amniotic fluid, show that at term about 30% of urinary 16α-hydroxyoestrone is of maternal origin while the values for 16-ketooestradiol and 16-epioestriol are each 20%.

SECTION C

METABOLISM/INTERCONVERSION OF FREE OESTROGENS *IN VITRO* AND *IN VIVO*

INTRODUCTION

Following the isolation of the ring D α-ketols and 16-epioestriol over the period 1955-1958 (1, 3, 4) considerable interest was evident in the interconversion of these and other steroids and in their relationship to oestriol (48-52). Certain related investigations were performed in the author's laboratory using both *in vivo* (human) and *in vitro* (human and other species) methods. Particular use was made of tissues from the laying hen (Gallus domesticus) in this work since the *in vivo* oestrogen metabolite interrelationships (both endogenous and exogenous) were being actively studied by Common *et al*. (53-57) in this species. Also, it had been noted that the hen produced 16-epioestriol as a major 16-oxygenated metabolite (58, 59). *In vitro* studies on chicken liver by Ozon and Breuer (60) appeared in 1965.

In addition to the above, some information was obtained regarding the formation of 2-methoxyoestrone using rat tissues *in vitro* as the sources of enzymes.

Finally a study of the metabolic fate of the synthetic oestrogen 17α-ethynyloestradiol-17β-3-cyclopentyl ether was performed in the human. This slow releasing precursor of oestrogenic activity, due to storage in and slow release from

adipose tissue (as studied in the rat; see reference 61), had attracted considerable attention as a potentially useful clinical drug (62-64).

Discussion of Published Work (Appendix - Section C)

a) 16-Oxygenated Steroids. In the late 1950's the biosynthesis of oestriol from oestradiol *in vitro* was a problem which had defied elucidation. Thus, although the former was recognized as a major metabolite in the human *in vivo*, several reports of its *in vitro* formation had been negated due to demonstrated inadequate purification of the product. In late 1958 and early 1959 three communications, in rapid succession, reported the production of oestriol from oestradiol by foetal human (65), rat (66) and laying hen (Mitchell and Hobkirk, *Biochem Biophys Res Commun* 1: 72, 1959) liver preparations. In 1965 Ozon and Breuer (60) demonstrated the 16α-hydroxylation of oestradiol by liver slices of chicken but also showed that 16β-hydroxylation, to form 16-epioestriol, predominated. In 1967 Raud and Hobkirk (*Biochem J* 103: 724, 1967) showed that the interconversion, 16-epioestriol $\rightleftharpoons$ oestriol was markedly in favour of the 16β hydroxy form in homogenates of laying hen liver. This appeared to be in some accord with the *in vivo* studies of Common *et al*. (53, 59) and demonstrated conclusively that in some species, at least, oestriol, although formed, need not be entirely an end product of metabolism as it virtually appears to be (apart from factors involving conjugation) in the human.

In studies along related lines, 16-ketooestradiol-^{14}C, made available by Dr. Mortimer Levitz, New York, was used as a substrate to demonstrate considerable species difference with respect to the stereochemical reduction of the 16-keto group by erythrocytes (Trachewsky and Hobkirk, J Biol Chem 239: 3683, 1964). Thus rat cells reduced the substrate in the β-configuration, yielding 16-epioestriol, while cat cells yielded almost exclusively oestriol (α-reduction). The physiological implications of these transformations are unknown and perhaps unimportant but it appeared that such a system might offer a useful model for study applicable to other tissues (e.g., liver) where conversions of this type are of considerable quantitative significance. An extension of the latter study, employing red cell haemolyzates, established an absolute requirement for NADP (NADPH) as a cofactor. Also, the conversions were shown to be reversible, the rat preparation catalyzing only 16-ketooestradiol $\rightleftharpoons$ 16-epioestriol, and the cat the interconversion, 16-ketooestradiol $\rightleftharpoons$ oestriol (Hobkirk, Nilson and Belenkie, Can J Biochem 43: 1893, 1965). Similar specificities have been demonstrated for 17-ketone and 17-hydroxyl groups of oestrone and oestradiol, respectively, in certain species (67) and Portius and Repke, in 1960 (68) had demonstrated the 16-ketooestradiol $\rightleftharpoons$ 16-epioestriol conversion in rat erythrocytes. However, after careful scrutiny of the latter report it was considered that insufficient information regarding degree of conversion and product identity was provided. In 1959 Breuer

(69) published data on the conversion of 16-ketooestradiol to 16-epioestriol by human erythrocytes. To date no further published work has appeared on the topic of oxidoreduction at carbon-16 by red cell enzymes. The data obtained by the author support results of the study on avian metabolism *in vitro* in that they provide more information about the occurrence of enzymes, in certain species, capable of catalyzing conversion of the D ring of phenolic steroid triols.

Various interrelationships of 16-oxygenated phenolic steroids in the human *in vivo* have been studied in the author's laboratory (Hobkirk, *J Clin Endocr* 23: 279, 1963; Hobkirk, Nilsen and Purre, *Can J Biochem* 44: 1211, 1966). Attempts to establish endogenous levels of ring D α-ketols in non-pregnancy urine, and as metabolites of labelled oestradiol, probably yielded overestimates due to lack of knowledge regarding the effect of purification on small weights of these, and to interference by 2-hydroxy oestrogens now known to be major metabolites of oestradiol in the human (70). Nevertheless, several interesting points were elucidated regarding interconversion of 16-oxygenated compounds. It was evident that 16-ketooestradiol was not a precursor of 16α-hydroxyestrone although it gave rise to oestriol and 16-epioestriol, in agreement with Levitz *et al.* (50). Also, oestriol was metabolized only slightly to 16-ketooestradiol and 16-epioestriol, once more in agreement with Levitz (49), whereas 16-epioestriol appeared to be converted to a greater extent to oestriol, as

well as to 16-ketooestradiol, suggesting a more marked biological conversion of the 16β, 17β-dihydroxy grouping than of the 16α, 17β-dihydroxy structure in the human (in this case the male). Diczfalusy et al. (71) have recently shown that quantities of oestriol may be formed from 16-epioestriol by maternal tissues of the pregnant woman. The present author's data also demonstrated that both labelled 16-epioestriol and 16-ketooestradiol gave rise in vivo to small amounts of 16β-hydroxyoestrone suggesting that the latter, or some fraction of it, may arise via oxidation of preformed 16-oxygenated steroids rather than by, or in addition to, direct hydroxylation as for 16α-hydroxyoestrone production. This is in some agreement with certain data of Breuer et al. (72, 73) but at variance with those of Fishman et al. (74, 75).

Breuer et al. (51) have devoted considerable attention to 16-ketooestrone (i.e., the 16,17-dione) as a possible intermediate in many of these interconversions. However, although the latter steroid has been claimed as a urinary metabolite (76) its existence is currently in question. A short communication from the author's laboratory (Lucis and Hobkirk, Steroids 1: 678, 1963) showed a product with the properties of 16-ketooestrone to be formed from 16-ketooestradiol incubated with human uterine fibroid tissue. The significance of this is not known.

(b) 2-Methoxyoestrone. Cell-free systems (usually microsomes plus supernatant) of rat liver tissue were demonstrated to

hydroxylate oestrone at position 2, to methylate the 2-hydroxyl group, thus forming 2-methoxyoestrone, and to reduce the latter to some degree to 2-methoxyoestradiol (Lucis and Hobkirk, Steroids 2: 669, 1963). The methoxylation could be inhibited by oestriol, 3,4-dihydroxy benzoic acid and chlorogenic acid.

(c) 17α-Ethynyloestradiol-17β-3-cyclopentyl ether. At the time of study this was known to be a long-acting oestrogen which, at least in the rat, was stored in adipose tissue prior to being released over a prolonged period (61). A study was made (Williams, Layne, Hobkirk, Nilsen and Blahey, Steroids 9: 275, 1967) of urinary and tissue metabolites of the above-mentioned compound labelled with ^{14}C in the cyclopentyl ether group and ^{3}H in the steroid. Excretion in the urine following a single oral dose was prolonged - 50 to 100 days. ^{3}H and ^{14}C were excreted in a non-uniform manner and three main metabolites were detected. One was identified as ^{3}H-ethynyloestradiol while a second, more polar compound, containing only ^{3}H, was probably a hydroxylated form of the latter (see reference 77). A third metabolite contained ^{3}H and ^{14}C and was thought to be a hydroxylated form of the administered compound (see reference 77). Virtually no unchanged 17α-ethynyloestradiol-3-cyclopentyl ether was excreted in the urine. A compound similar to it was recovered from adipose tissue but no radioactivity could be measured in a variety of other tissues including blood, ovary, ovarian tube, endometrium and uterine wall. Thus, in the human the long-term activity of this synthetic oestrogen is probably

due to its slow release from adipose tissue with the production of active oestrogen such as 17α-ethynyloestradiol.

SECTION D

URINARY OESTROGEN CONJUGATE PATTERNS BASED MAINLY ON DIRECT HYDROLYTIC PROCEDURES

INTRODUCTION

With the availability of more or less specific methods for hydrolysis of steroid glucosiduronates (β-glucuronidase) and sulphates (solvolysis) it became possible, and advisable, to investigate the 'conjugation pattern' of oestrogen metabolites in human urine (both pregnant and non-pregnant). Such studies had, of course, been performed to some extent, over the years (78-80), but little or nothing was known regarding, for example, conjugation of the phenolic steroid ring D α -ketols until Smith and Kellie's observations were published in 1967 (81). Initial work on the conjugation of the classical oestrogens in glucosiduronate and sulphate forms had resulted in very variable results (79, 82, 83) so that it was considered worthwhile to follow this avenue of investigation, particularly in view of the increasing interest in steroid sulphates in biochemistry and metabolism (84, 85).

Discussion of Published Work (Appendix - Section D)

Conjugation patterns in the non-pregnant human female were established following intravenous administration of trace doses of ^{3}H-oestradiol (Hobkirk, Nilsen and Blahey, J Clin Endocr 29: 328, 1969). Of the urinary ^{3}H extractable after successive treatment with β-glucuronidase and solvolysis,

some 10-15% usually corresponded to 'sulphate', i.e., was solvolyzed. Of this, a large fraction, in some cases virtually all, appeared to be oestrone sulphate. In only a few instances was it possible to identify with certainty non-ketonic steroids or ring D α -ketols which had been conjugated in a sulphate form. In one particular subject a large percentage of extractable urinary ^{3}H (some 50%) behaved like sulphate. It should be pointed out here that because of the successive β-glucuronidase/solvolysis treatment, steroids with more than one hydroxyl group, conjugated with both glucuronic acid and sulphuric acid, would end up in the 'sulphate' (solvolyzed) fraction. In the above-mentioned subject all of the steroid fractions sought were identified partially in a solvolyzable form and oestrone-3-sulphate was shown by direct means to be the main single component so conjugated. The significance of this is not clear, nor is the question of how intimately such a feature is concerned with changes (within the normal range) in renal function. Endogenous oestrone in human females was also measured in glucosiduronate and sulphate forms and on the average the latter were calculated to be 32 and 37%, respectively, of total urinary oestrone at days 14 and 22 of the normal cycle. These values, however, include the elevated oestrone sulphate found in the subject discussed above. However, even with omission of these results, 25 and 28% are the values obtained. Thus urinary oestrone-3-sulphate may be a more quantitatively significant urinary metabolite in the

human than earlier indicated.

Because of the hydrolytic sequence employed the above study did not differentiate between sulphates and sulpho-glucosiduronates (see above). Further data became available on such endogenous conjugates in human pregnancy urine although still in a somewhat indirect fashion (Tan, Anuman-Rajadhon and Hobkirk, Clin Chim Acta 31: 783, 1971). Even so it was concluded that conjugates other than monoglucosiduronates of the phenolic steroid ring D α-ketols (as a percentage of the total individual steroid fraction) were greater than for oestriol. However, there was little or no 16 α-hydroxyoestrone sulphate present although considerable amounts of the sulphoglucosidur-onate of this steroid were found, and significant levels of both monosulphate and sulphoglucosiduronate of 16-keto-oestradiol were measurable. Whether this reflects a lack of sulphurylation of 16α-hydroxyoestrone, or a feature of renal function, is not known at present. A recent paper by Ahmed and Kellie (85a) indicates that the sulphoglucosiduronate of 16α-hydroxyoestrone accounts for some 20% of total conjugates of this steroid in late pregnancy; in good agreement with Tan et al. The studies of Smith and Kellie (81) and those of Bugge, Nilsen, Metcalfe-Gibson and Hobkirk (Can J Biochem Physiol 39: 1501, 1961), together with the above, show the necessity for taking the monosulphate and double conjugate groupings into consideration when hydrolyzing ring D α-ketol conjugates in pregnancy urine.

Unpublished Data Related to the Above

(a) Serial analysis of ring D α-ketols in pregnancy. Figs 2 and 3 show excretion patterns for 16α-hydroxyoestrone and 16-ketooestradiol in the 'glucosiduronate' and 'non-glucosiduronate' forms following successive β-glucuronidase incubation and solvolysis. The curves for all forms appear to follow closely that for urinary oestriol, with the typical increase in rate of excretion at about 30 or more weeks. Adlercreutz and Luukkainen (39) have now presented similar data for 'total' urinary levels of these steroids using gas liquid chromatography combined with mass spectrometry.

(b) Specific radioactivities of conjugated oestrogen metabolites in pregnancy urine after a single intravenous injection of oestradiol-6,7-^{3}H. In an earlier publication (Hobkirk and Nilsen, J Clin Endocr 26: 625,1966) it was concluded, on cursory examination, that the specific activities of 'glucosiduronates' and 'sulphates' (or non-glucosiduronates) of any single oestrogen metabolite in such experiments were, to all intents and purposes, the same, and values were therefore given for 'combined' conjugated forms. The results of two further experiments, in which specific activities were established by a double isotope procedure following successive β-glucuronidase hydrolysis and solvolysis, are shown in Table 4. As in the earlier studies the values for the ring D α-ketols were much closer to those of oestrone than to those of oestriol. The values for the 'non-glucosiduronate' (sulphate plus

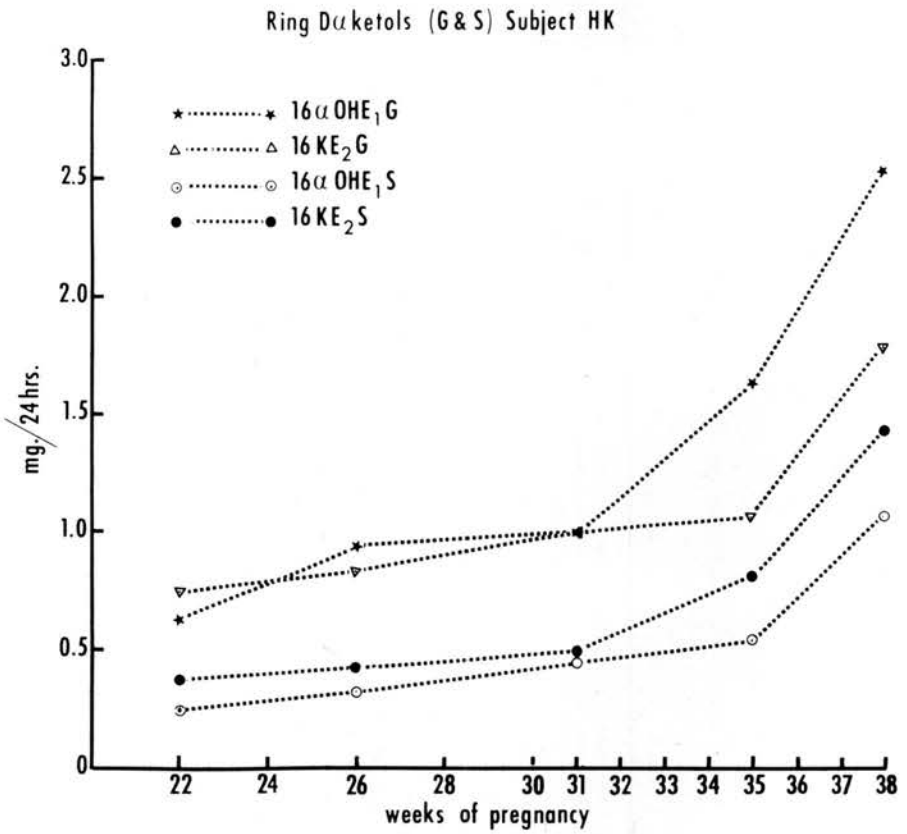
Ring Dα ketols (G & S) Subject HK
16α OHE1G
16 KE2G
16α OHE1S
16 KE2S
mg./24 hrs.
3.0
2.5
2.0
1.5
1.0
0.5
0
22
24
26
28
30
31
32
33
34
35
36
37
38
weeks of pregnancy

Figure 2

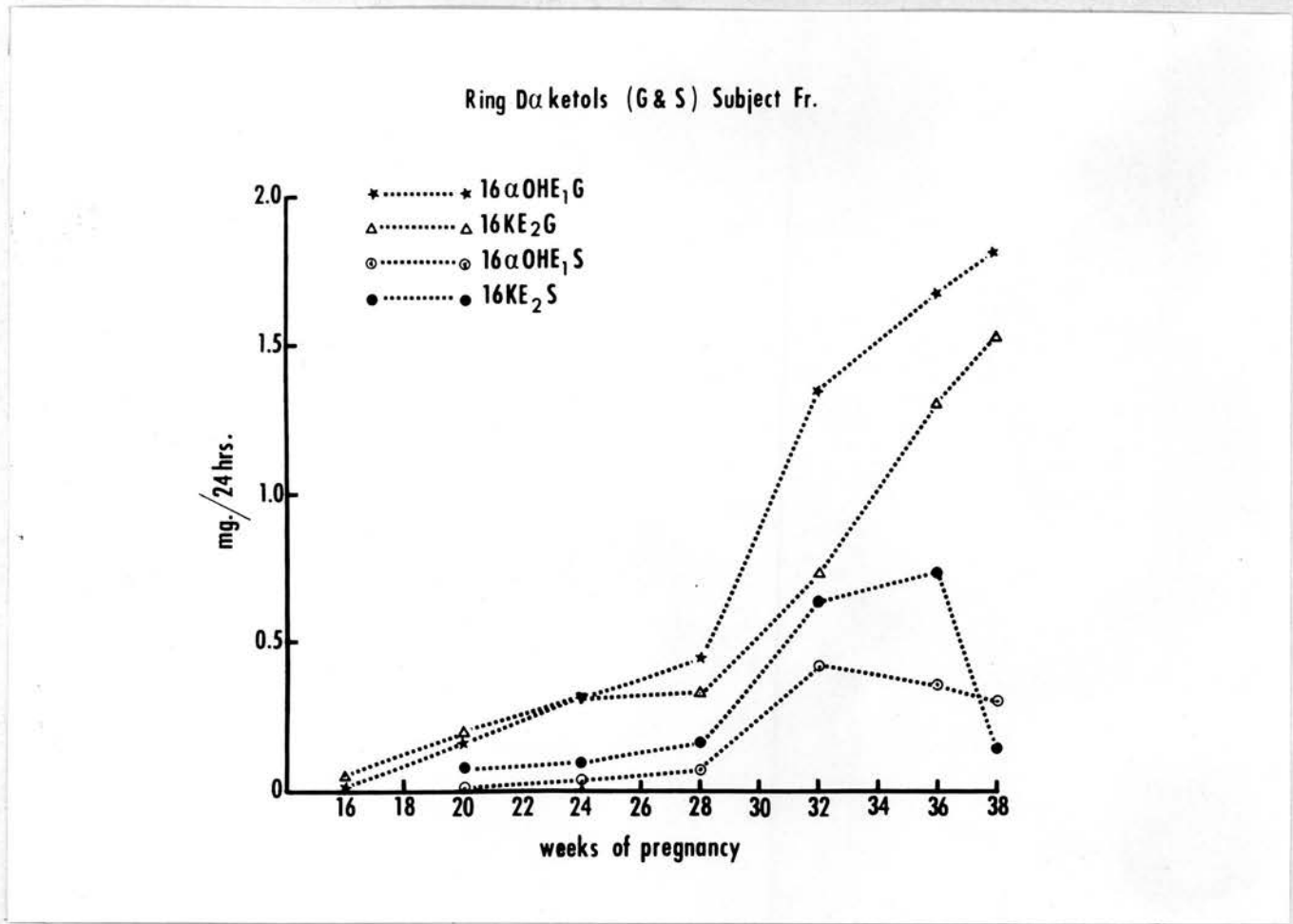
Ring Dα ketols (G & S) Subject Fr.
16αOHE1G
16KE2G
16αOHE1S
16KE2S
2.0
1.5
1.0
0.5
0
mg./24 hrs.
16
18
20
22
24
26
28
30
32
34
36
38
weeks of pregnancy

Figure 3

TABLE 4

Specific Radioactivities (dpm/ug) of Six Urinary Metabolites of Intravenously Administered ^{3}H-Oestradiol (25.3 x 10^{6} dpm) of Two Pregnant Women in the Third Trimester (results refer to free steroids).

Subject	2-Methoxy-Oestrone		Oestrone		Oestradiol		16α-Hydroxy-Oestrone		16-Keto-Oestradiol		Oestriol	
	G*	S**	G	S	G	S	G	S	G	S	G	S
1	234	346	272	348	303	696	179	171	147	124	27	39
2	305	308	411	505	444	710	284	322	213	241	47	58

* Steroid released by β-glucuronidase.

** Steroid released by solvolysis <u>after</u> β-glucuronidase incubation.

sulphoglucosiduronate forms) were somewhat similar to or greater than those for the glucosiduronates. The differences which occurred were not great enough to suggest highly differing percursor or pool origins, except perhaps in the case of oestradiol. In that instance the higher values for the 'sulphate' could have been due to the rapid excretion of a small percentage of the injected ^{3}H-oestradiol which had become conjugated without mixing with the main body pool.

(c) Conjugation of urinary oestrogen metabolites in pregnant and non-pregnant subjects. Published data (see Section D, above) and additional unpublished material are collected in Table 5. The main (in a quantitative sense) metabolites only are presented. Broadly speaking, it is seen that the degree of conjugation of oestrone as its sulphate, although higher on the average in its endogenous form in pregnancy than as a metabolite of injected ^{3}H-estradiol in the non-pregnant state, covers a similar wide range. Also, in the non-pregnant state the endogenous pattern is similar, although the mean value is higher than for the corresponding exogenous value. The values for 16α-hydroxyoestrone and 16-ketooestradiol are virtually identical in the pregnant (endogenous) and non-pregnant (exogenous) groups. The same is true of oestriol. Thus it might be claimed that the production of vastly increased amounts of these steroids in pregnancy does not lead to a situation where the enzymes and tissues involved in their conjugation and distribution are unable to handle them, at least as based on

TABLE 5

Percentages (means and ranges) of Four Urinary Phenolic Steroids Conjugated as 'Non-Glucosiduronate' (sulphate + sulphoglucosiduronate) in Pregnant and Non-pregnant Women.

Origin of Urinary Metabolites	Oestrone 23 subjects	16α-Hydroxy-oestrone 28 subjects	16-Keto-oestradiol 28 subjects	Oestriol 28 subjects
Non-pregnant; Metabolites of ^{3}H-oestradiol	15 (1-65)	20* (3-33)	33* (6-61)	4** (1-8)
Non-pregnant; endogenous metabolites	30 (1-71)	-	-	-
Pregnant; endogenous metabolites	25 (1-56)	21 (6-42)	37 (14-81)	4.5 (1-14)

* In only 8 out of 28 were these steroids definitely identified as being in the 'non-glucosiduronate' forms. The values given are for these 8 subjects.

** In only 7 out of 28 was oestriol definitely identified in the 'non-glucosiduronate' form.

urinary patterns. However, where the ring D α-ketols and oestriol are concerned it was possible to definitely identify the steroids in their 'non-glucosiduronate' forms in only a small number of the non-pregnant subjects, i.e., 7 or 8 out of 28 (see Table 5). Had firm data been obtained for all these (e.g., by injecting a larger dose of ^{3}H-oestradiol) it is quite possible that the average 'non-glucosiduronate' percentage would have been lower in these latter cases. The wide variation between individuals should also be borne in mind, as shown in Table 5.

SECTION E

FORMATION, METABOLISM, INTERCONVERSION OF OESTROGEN SULPHATES IN SPECIES OTHER THAN THE HUMAN *IN VITRO* AND *IN VIVO*

INTRODUCTION

With the increasing interest in steroid sulphates, certain of which are now considered to be products of secretion (86-88) and intermediates in biosynthetic processes (84, 85, 89, 90) rather than, or as well as, 'detoxification' products, several aspects of this field of interest appeared suitable for study. Thus, some consideration has been given to the effect of steroid structure on enzymatic sulphurylation, as well as to the age of the animal (rat) from which tissue was obtained for *in vitro* work. Since the *in vivo* pattern of oestrogen metabolism in the chicken has been particularly well established (53-59), certain aspects of sulphate conjugate metabolism were investigated using this species both *in vitro* and *in vivo*. The usefulness of this latter study was greatly increased by the knowledge that sulphurylation may represent the sole type of conjugation which occurs in the chicken, at least as reflected in the products of urinary excretion (10a).

Discussion of Published Work (Appendix - Section E)

A soluble sulphurylating enzyme system from rat liver was shown to display greater activity toward phenolic steroids possessing ring D hydroxyl groups of the β-orientation (e.g., 17β -oestradiol, 16-epioestriol and 16-ketooestradiol-17β) than

towards steroids with an α-orientated group in ring D (e.g., oestriol); Raud and Hobkirk (Can J Biochem 44: 657, 1966). The disulphate conjugate was formed from oestradiol. In the foetal rat at term no sulphurylation of oestrone or oestradiol was observed with only minimal sulphurylation of dehydroisoandrosterone. Even at 10 days of age no activity was displayed towards oestrone and as late as 30 days the observed conjugation of each of the above three steroids was far from the adult level.

A high speed supernatant fraction of chicken liver was many times more active in sulphurylation than were similar preparations of oviduct and vagina from the same species (Raud and Hobkirk, Can J Biochem 46: 749, 1968). Moreover, the latter system, in each tissue, demonstrated a much greater activity towards the phenolic steroids than to dehydroisoandrosterone. This represents a curious finding since sulphurylation of 3β-OH, Δ^5 steroids is generally considered to be an efficient biochemical mechanism in a variety of tissues from many species, e.g., rat liver (Raud and Hobkirk, Can J Biochem 44: 657, 1966). These results appear to be in good agreement with the ability of the chicken to sulphurylate oestrogens in vivo (10a).

Incubation of ^{14}C-oestrone and ^{3}H-oestradiol-3-sulphate with liver homogenates from laying hens (Raud and Hobkirk, Can J Biochem 46: 759, 1968) furnished the following information: (a) There is interconversion in this system between free

oestrone and oestradiol with production of the former steroid predominating, just as for almost all in vitro and in vivo situations thus far studied. (b) There is interconversion, apparently in the intact conjugated form, of oestrone-3-sulphate and oestradiol-3-sulphate, once more with the 17-keto form predominating. This supplements earlier information regarding direct interconversion of intact steroid conjugates in other species and systems and may also be relevant to the later in vivo work of Mathur (91) who showed in 1969 that, in this species, metabolism of intact oestrogen conjugates occurred.

The identification of disulphates of phenolic steroids as metabolites in the urine of the laying hen (10a, 91) suggested the necessity for studying the metabolism of labelled oestradiol-3,17-disulphate in vivo in the same species. The results of such a study (Mathur, Common and Hobkirk, Steroids 14: 389, 1969) indicated a very poor recovery of the label in the urine. The sole metabolite identified was oestradiol-17-sulphate. Thus a partial picture was obtained showing the possibility of removal of the 3-sulphate group from the diconjugate.

SECTION F

METHODOLOGY FOR THE SEPARATION OF STEROID CONJUGATES

INTRODUCTION

Part of the foregoing work (Sections B and D) has yielded data derived from experiments which either did not attempt to differentiate between conjugated forms of steroids or which relied almost wholly on the specificity (occasionally doubtful) of enzymatic hydrolysis and/or solvolysis to cleave various crude mixtures of glucosiduronates, sulphates and sulpho-glucosiduronates in urine or extracts thereof. There is clearly a limit to such methodology, particularly where the metabolism of steroid conjugates is to be investigated. Considerable advances have been made in the separation of intact steroid conjugates (11, 12, 18, 85, 92, 93) although many of these techniques are not without their problems. Moreover, a relatively simple and efficient separation of oestrone-3-glucosiduronate, oestradiol-3-glucosiduronate and oestradiol-17-glucosiduronate, did not appear to have been achieved. A better understanding of the *in vitro* biosynthesis of the labelled forms of these conjugates (94-97) enabled standard compounds to be prepared thus rendering the overall problem much easier. A workable separation procedure involving simple gradient elution of DEAE-Sephadex columns was devised, taking advantage of the pioneering studies of Hahnel (11, 12). Attempts were also made to apply this to the separation of certain C_{19} neutral steroid conjugates.

Discussion of Published Work (Appendix - Section F)

A relatively simple method involving linear concentration gradients of NaCl in H_2O allowed separation for the first time of a number of conjugates including the 3-glucosiduronates and 3-sulphates of oestrone and oestradiol, and the 17-glucosiduronate, the 3-sulphate-17-glucosiduronate and the 3,17-disulphate of oestradiol (Hobkirk, Musey and Nilsen, Steroids 14: 191, 1969). It should be noted that the pioneering work of Hahnel (11) did not result in the same efficient separations obtained in the present author's laboratory, particularly between the 3-glucosiduronates of oestrone and oestradiol and even between sulphates and 16- or 17-glucosiduronates. In order to separate the sulphates from glucosiduronates Hahnel resorted to a very complex gradient (12). During the preparation of the various labelled conjugates in the present work it was shown possible to produce oestradiol-3-sulphate-17-glucosiduronate from oestradiol-17-glucosiduronate by the sulphurylating enzyme system in guinea pig liver in a manner similar to that described by Levitz et al. (95) for oestriol-3-sulphate-16-glucosiduronate. This oestradiol conjugate had not previously been reported as a biosynthetic product but its chemical synthesis had been described (98).

A refinement of the procedure for the separation of the 3-glucosiduronates of oestrone and oestradiol and the 17-glucosiduronate of oestradiol was subsequently reported (Hobkirk and Nilsen, Anal Biochem 37: 337, 1970). This

involves two separate NaCl concentration gradients on DEAE-Sephadex with a Celite column partition chromatographic step inserted between them. This procedure has been extensively and successfully employed in the author's laboratory to study the detailed metabolism of the glucosiduronates and glucosides of oestrone and/or oestradiol (see Sections G and H, below). These procedures, or modifications of them, have been or are now being successfully used in a variety of studies in the laboratories of D. S. Layne (University of Ottawa; personal communication), K. F. Stoa, Bergen, Norway (99) and D. C. Collins et al., Atlanta, Georgia (100, 101). Shortly after the present author's first paper on the subject (Hobkirk, Musey and Nilsen, Steroids 14: 191, 1969) two publications appeared on the use of a number of Sephadex preparations in the attempted separation of all the possible conjugated urinary metabolites of injected ^{14}C-oestradiol in the human (102, 103). These data once more demonstrated the usefulness of Sephadex in this field but the complexity of the study resulted in a failure to identify the majority of the large number of separated or partially separated peaks.

Attempts to apply the gradient elution technique to the problem of neutral steroid separation has been only partially successful (Hobkirk and Davidson, J Chromatog 54: 431, 1971). It resulted in the separation of the sulphates of testosterone and dehydroisoandrosterone from each other and from the glucosiduronates of these two steroids; the glucosiduronates were

not, however, resolved. The free steroids were easily separated (as one peak) from the conjugates.

SECTION G

METABOLISM OF OESTRONE AND OESTRADIOL GLUCOSIDURONATES IN THE HUMAN IN VIVO

INTRODUCTION

Although considerable data have been accumulated as to the metabolism of oestriol conjugates in the human by Levitz *et al.* (85, 104), Diczfalusy *et al.* (105-107) and Slaunwhite *et al.* (92, 108, 109) very little of note had been published regarding the metabolism of glucosiduronates of oestrone and oestradiol prior to the present author's work. Indeed, it would seem that only one publication had appeared, that being on the subject of the metabolism of oestrone-3-glucosiduronate in pregnancy (110). It was shown that a very small amount of the latter conjugate was transferred intact from foetus to mother and that in the foetus a limited degree of 16-hydroxylation occurred directly to yield oestriol and/or 16α-hydroxyoestrone glucosiduronates. In addition, a small degree of direct reduction of oestrone-3-glucosiduronate to oestradiol-3-glucosiduronate was indicated. In the same study the high rate of urinary excretion of the isotope following intravenous injection of singly labelled oestrone-3-glucosiduronate into pregnant women was taken to reflect a general lack of metabolism of the conjugate. During the course of the present author's work it was shown by Roy and Slaunwhite (111) that oestrone-3-glucosiduronate and oestradiol-3-glucosiduronate were interconverted in their conjugated forms

by a human placental dehydrogenase enzyme system *in vitro*. However, the use of considerable concentrations of cofactors made it difficult to draw conclusions regarding the preferred direction of this interconversion.

The availability of a procedure for the direct separation and purification of oestrone and oestradiol glucosiduronates (see Section F, above) rendered possible a study of the metabolism of these conjugates in the human female. The reasons for such a study included the basic lack of information in this area, the necessity of knowing whether these glucosiduronates are indeed real 'end products of oestrogen metabolism', and a need to understand whether the same conjugates necessarily represent 'unique metabolites' of secreted oestrogens, particularly with respect to measurement of production and secretion rates from urinary metabolites of injected hormones (112-114). Besides these points, the form(s) of the naturally-occurring oestradiol glucosiduronate(s) was (were) unknown; i.e., whether there existed the 3-mono, 17-mono, or 3,17-diglucosiduronate form, or indeed, any combination of these.

Discussion of Published Work (Appendix - Section G)

The commercial availability of oestradiol-6,7-^{3}H-17-glucosiduronate made possible a comparison of the metabolism of this conjugate with that of unconjugated ^{14}C-oestradiol (Hobkirk and Nilsen, *Steroids* 13: 679, 1969). This study was performed using conventional hydrolytic techniques followed by identification of the various free steroidal metabolites rather than by

preliminary separation of the conjugates since the latter procedure was not yet available. Thus little information on specific conjugate structure was obtained for the urinary metabolites. However, a great difference between the metabolism of the free and conjugated oestradiol was apparent. From the latter, little besides the glucosiduronates of oestrone and oestradiol were excreted whereas administered oestradiol gave rise to the usual multitude of metabolites including major quantities of conjugated 16-hydroxy steroids, such as oestriol. The formation of oestrone glucosiduronate as a major metabolite of oestradiol-17-glucosiduronate confirmed the removal of the 17-glucuronyl group without, however, leading to release of oestradiol (or oestrone) into the main body pool, otherwise one would have expected to find similar metabolites from the conjugated and unconjugated percursors (i.e., oestradiol-17-glucosiduronate and oestradiol). The possible presence of oestradiol-3-sulphate-17-glucosiduronate as a metabolite of oestradiol-17-glucosiduronate was also suggested.

The above data necessitated the identification of the urinary conjugated metabolites of oestradiol-17-glucosiduronate and this was undertaken with the aid of the gradient elution procedure described in Section F. Oestrone-3-glucosiduronate, oestradiol-3-glucosiduronate and oestradiol-17-glucosiduronate were identified as the main metabolites, together with small amounts of oestradiol-3-sulphate-17-glucosiduronate and oestrone-3-sulphate (Hobkirk and Nilsen, Steroids 14: 533, 1969).

The relative amounts of these were not established with certainty since identification was achieved at the expense of quantitation. The route of conversion was not established but it was suggested that a possible intermediate could be oestradiol-3,17-diglucosiduronate, as follows: Oestradiol-17-glucosiduronate → oestradiol-3,17-diglucosiduronate → oestradiol-3-glucosiduronate → oestrone-3-glucosiduronate. For this to occur the facile conversion of oestradiol-3-glucosiduronate to oestrone-3-glucosiduronate should be demonstrable. With this in mind the latter two conjugates were prepared, in the author's laboratory, containing ^{3}H in the steroidal moieties and ^{14}C in the glucuronic acid residues. A study of the metabolism of these, after their intravenous injection into normal women (Hobkirk and Nilsen, Steroids 15: 649, 1970), revealed direct interconversion within a relatively short time interval, this being markedly in favour of oestrone-3-glucosiduronate production in a ratio of about 4:1. Urinary excretion of the labels was very rapid, in agreement with Zuconni et al. (110) and little additional radioactivity was excreted beyond 6 hr after injection. The latter consisted solely of ^{3}H and had apparently arisen via deconjugation (hydrolysis), interconversion of free oestrone and oestradiol, and reconjugation with endogenous, unlabelled, glucuronyl groups. No oestradiol-17-glucosiduronate was excreted in the labelled form and little or no additional metabolites were detected. These findings are not inconsistent with the possible occurrence of the scheme

outlined above for the conversion of oestradiol-17-glucosiduronate to oestrone-3-glucosiduronate, however, the diglucosiduronate form of oestradiol has not yet been detected in any situation.

Since there is little, if any, evidence for deconjugation of oestrogen glucosiduronates, in the human, at sites other than in the intestine (see studies on oestriol glucosiduronates; references 92, 108), and in view of the partial deconjugation of intravenous oestradiol-17-glucosiduronate and the 3-glucosiduronates of oestrone and oestradiol found to occur (see above), the labelled forms of these compounds were administered orally to women (Hobkirk, Nilsen and Musey, Un Med Canada 100: 449, 1971). Almost the entire urinary radioactivity could be accounted for as oestrone and oestradiol-3-glucosiduronates after the ingestion of oestradiol-17-glucosiduronate. Very small amounts of oestriol glucosiduronate(s) was detected while the administered conjugate was apparently absent in its unchanged form. Orally administered oestrone-3-glucosiduronate, containing ^{3}H in the steroid and ^{14}C in the glucuronic acid group yielded little other than ^{3}H-labelled oestrone-3-glucosiduronate and oestradiol-3-glucosiduronate in the urine, indicating efficient deconjugation and reconjugation. This further suggested a hydrolysis-absorption-conjugation mechanism at the gut level. In these experiments a considerable delay of at least 3 hr occurred in urinary excretion of the radioactivity. Over that period a very small amount of isotope (^{3}H plus ^{14}C)

appeared followed by a progressive, but slow, excretion of ^{3}H alone up to at least 72 or 96 hr. This was suggestive of a rate-limiting step at some point in the overall absorption (including deconjugation and reconjugation) mechanism.

Of paramount importance in these studies has been the identification of the naturally-occurring urinary conjugates of oestradiol. Labelled oestradiol was administered intravenously or orally to normal women, and the urinary conjugates were investigated (Hobkirk and Nilsen, J Clin Endocr 32: 779, 1971). Within 2 hr of intravenous injection, labelled oestradiol-17-glucosiduronate exceeded the 3-glucosiduronate by a factor of more than 4. Thereafter virtually no further 17-glucosiduronate was excreted whereas the 3-glucosiduronate increased cumulatively over a 24 hr period and approached the level of the former conjugate at that time. It should be noted that Hahnel, in 1967, claimed these two compounds, in their endogenous forms, to be present in pregnancy urine in a ratio of about unity (115) and in 1970 showed the same to be true of the two conjugates as urinary metabolites of labelled oestradiol injected during pregnancy (116). After ingestion of oestradiol no labelled oestradiol-17-glucosiduronate was detectable in the urine but the 3-glucosiduronate of oestradiol (besides that of oestrone) was excreted in an increasing cumulative fashion over at least 24 hr. It is noteworthy that, following oral ^{3}H-oestradiol, urinary excretion of the label occurred without delay (i.e., within the limits that 2 hr was the first collection time;

compare reference 117). Thus it would seem logical to suggest that the delay in, and slow rate of, excretion, following upon ingestion of oestrogen glucosiduronates (see above) is related to the deconjugation, or hydrolytic step, prior to, or during, the absorptive process.

The above data established, among other things, the identity of the main urinary metabolites of oestradiol-17-glucosiduronate but yielded little information on precisely how these metabolites arose or in what sequence. In order to clarify these points, further work was carried out, and this resulted in a fairly firm understanding of the overall metabolism of oestradiol-17-glucosiduronate, particularly with respect to the role of an enterohepatic system (Musey, Green and Hobkirk, J Clin Endocr 35: 448, 1972). When ^{3}H-oestradiol-17-glucosiduronate was injected intravenously into normal females the excretion of ^{3}H was rapid over about 0-2 hr (20-40% dose) and then almost ceased over about 2-6 hr to be followed by a recommencement of excretion up to at least 72 hr. In 0-2 hr almost all of the label was in the form of oestradiol-17-glucosiduronate (presumably unchanged injected material) together with very small amounts of oestradiol-3-sulphate-17-glucosiduronate. These two compounds more or less disappeared from the urine thereafter, to be replaced by the 3-glucosiduronates of oestrone and oestradiol and very small amounts of the glucosiduronates of other steroids such as oestriol. When ^{3}H-oestradiol-17-glucosiduronate was injected as above

into women draining bile via T-tube the 0-3 hr urine contained the compounds found in 0-2 hr in the normal subjects and the 0-3 hr bile contained major amounts of oestradiol-3-sulphate-17-glucosiduronate and variable amounts of an as yet unidentified sulphoglucosiduronate but no detectable monoglucosiduronates of oestradiol or oestrone. Later samples of bile (up to 24 hr) contained very small amounts of ^{3}H, in the form of sulphoglucosiduronates, and urine from 3-6 hr onwards contained variable amounts of oestradiol- and oestrone-3-glucosiduronates. It is likely that these latter arose via escape of some bile into the intestine with subsequent metabolism of the sulphoglucosiduronate of oestradiol. Instillation of ^{3}H-oestradiol-3-sulphate-17-glucosiduronate into the duodenum of a normal subject resulted in a marked delay (some 6 hr) in urinary excretion of the label and then a rather slow excretion up to at least 96 hr. The labelled metabolite pattern was very similar to that found beyond 6 hr after intravenous injection of oestradiol-17-glucosiduronate into intact subjects; i.e., the 3-glucosiduronates of oestradiol and oestrone were by far the major compounds produced. It was also ascertained that a small fraction (ca 7%) of an intravenous dose of oestrone-6,7-^{3}H-3-glucosiduronate-^{14}C was excreted in the bile of a T-tube subject, apparently in contrast to the absence of oestradiol-17-glucosiduronate from the bile after intravenous injection of its labelled form.

These results explain the pathway of oestradiol-17-glucosiduronate to its major urinary metabolites oestradiol-3-glucosiduronate and oestrone-3-glucosiduronate as being via biliary oestradiol-3-sulphate-17-glucosiduronate in the small intestine. There is also agreement between these data and those obtained for the metabolism of orally-administered oestradiol-17-glucosiduronate and oestrone-3-glucosiduronate (Hobkirk, Nilsen and Musey, Un Med Canada 100: 449, 1971) with regard to the delay in intestinal absorption and the absence of urinary oestradiol-17-glucosiduronate as an enteric metabolite. Moreover, the cessation of excretion of urinary labelled oestradiol-17-glucosiduronate within 2 hr after intravenous injection of ^{3}H-oestradiol to normal women (Hobkirk and Nilsen, J Clin Endocr 32: 779, 1971) can possibly be explained by synthesis and biliary excretion of oestradiol-3-sulphate-17-glucosiduronate which, by a hydrolysis-absorption-reconjugation mechanism would contribute, at least partly, to the continuing urinary excretion of labelled oestradiol-3-glucosiduronate (and oestrone-3-glucosiduronate) up to at least 24 hr after injection of the free steroid. Alternatively, the urinary pattern of oestradiol-17-glucosiduronate from precursor oestradiol could reflect conjugation in (for example) the kidney, with consequent rapid excretion. Kirdani et al. (118) have recently shown human kidney to be capable of conjugating oestriol with glucuronic acid at carbon 16 whereas oestrone was scarcely conjugated under the same conditions. Recent unpublished results from the

present author's laboratory show that intravenously injected ^{3}H-oestradiol in the human gives rise to urinary ^{3}H-oestradiol-17-glucosiduronate within 10 minutes of injection. Excretion of the latter conjugate virtually ceases within about 60 minutes. If 17-glucuronidation is indeed a renal activity there is little need to invoke oestradiol-3-sulphate-17-glucosiduronate (via oestradiol-17-glucosiduronate) as a major biliary metabolite of blood-borne oestradiol or as a major precursor of urinary oestrone-3-glucosiduronate and oestradiol-3-glucosiduronate in the human. However, until more definitive experiments can be performed, the exact nature of the routes of formation and metabolism of oestradiol-17-glucosiduronate must remain open to speculation.

Metabolism of Oestrone and Oestradiol Glucosiduronates - General Discussion

The data in Section G of this thesis establish, for the first time, although not entirely completely, the inter-relationships of the naturally-occurring glucosiduronates of oestrone and oestradiol. Apart from the work of Hahnel (115, 116) there has been little to suggest the number of glucosiduronate forms of oestradiol which may contribute to the release of free oestradiol during incubation of urine or urinary extracts with β-glucuronidase. It is quite evident from the present author's studies that both possible mono-glucosiduronates of oestradiol occur and that these may have different biosynthetic pathways; moreover they may exhibit

entirely different patterns of metabolism. Estrone-3-glucosiduronate, in common with oestradiol-3-glucosiduronate, could have several precursors and almost certainly more than one tissue of origin. Such information may complicate calculations of production or secretion rates of oestrogens where urinary glucosiduronates are considered as more or less direct metabolites of the secreted hormone(s). The situation becomes even more complex when it is considered that both oestradiol and oestrone may be secreted and, moreover, since the sulphates of the latter two oestrogens might conceivably play a role as precursors of the glucosiduronates. It may very well be, on the basis of the information presented by the author's laboratory that oestradiol-17-glucosiduronate is a unique metabolite of oestradiol, formed directly from the parent hormone.

One can speculate endlessly upon the relationship of glucosiduronate conjugation and the role of such oestrogen conjugates (if any) under physiological conditions. One particular area which appears worthy of attention concerns the presence of considerable amounts of highly polar oestrogen conjugates in human bile. According to the author's work it would appear that in order, for example, for biliary excretion of oestradiol-3-sulphate-17-glucosiduronate, or other conjugate, to lead to intestinal reabsorption of the oestrogen, a free, or possibly protein bound, form of the steroid (partly oxidised to oestrone) is probably an intermediate, perhaps in the gut wall.

Thus 'potentially-active' hormone might be delivered in this fashion to the intestine where it could conceivably play a role in maintenance of the circulatory system therein. This, of course, is merely speculation but it does suggest a reason for enquiry into the overall handling of these steroid conjugates in the human subject.

SECTION H

METABOLISM OF OESTROGEN GLUCOSIDES IN THE HUMAN IN VIVO

INTRODUCTION

Conjugates other than glucosiduronates and sulphates have been detected or identified in biological materials from time to time. Within recent years much attention has been accorded the N-acetylglucosaminides (119) and glucosides (120) of phenolic steroids. These compounds have been shown to occur particularly in the rabbit. The sole evidence for the occurrence of steroid glucoside biosynthesis in the human has been advanced by Layne et al. (121). These workers showed that human liver and kidney tissues, in vitro, are capable of catalyzing the transfer of glucose from adenosine diphosphate glucose to the 17-hydroxyl group of 17α-oestradiol. Although such transfer appears to be specific for the 17α-orientated hydroxyl group of oestradiol it was deemed worthwhile to study the metabolism of the 3- and 17-monoglucosides of 17β-oestradiol in the human so as to compare with the metabolism of the closely related (structurally) glucosiduronates, as in Section G, above. In addition, the metabolism of 17α-oestradiol-17-glucoside was studied since, at least in vitro, it can be formed in the human.

Discussion of Published Work (Appendix - Section H)

17β-Oestradiol-6,7-^{3}H-3-β-D-glucopyranoside-^{14}C (generally labelled) was synthesized and injected intravenously into human females (Hobkirk, Nilsen, Williamson and Layne, J Clin Endocr 32:

476, 1971). A rapid excretion of some 20% of each of the administered labels occurred within a 2 hr period, this representing, almost entirely, unchanged injected conjugate. Even within this time, however, a small amount of oestrogen monoglucosiduronate, containing only the ^{3}H label was excreted. Beyond 2 hr only ^{3}H-labelled metabolites were found, these reflecting in pattern the metabolism of free oestradiol. Thus it appears that by far the greater part of oestradiol-3-glucoside is hydrolyzed at the glucosidic linkage with release of oestradiol which in turn is metabolized in the same manner as circulating oestradiol. This is very different from the metabolism of oestradiol-3-glucosiduronate (Section G) and may relate to a greater ease of transport of the uncharged conjugate (glucoside) into cells and/or to the relative distribution of β-glucosidase and β-glucuronidase enzymes in the human.

When oestradiol-6,7-^{3}H-17-β-D-glucopyranoside was injected intravenously into normal women about 5% of the ^{3}H dose was excreted in the urine within 2 hr (Hobkirk, Nilsen, Williamson and Layne, J Clin Endocr 34: 690, 1972). In that time, in two subjects, 1.9 and 0.7% of the dose was identified as unchanged monoglucoside and 4.4 and 2.5% of the dose as a double conjugate of oestradiol with glucose at C-17 and glucuronic acid at C-3. Over the approximate period 2-6 hr after injection very little ^{3}H was excreted, then the label reappeared and excretion continued up to at least 72 hr. Within the approximate time interval 6-48 hr by far the main metabolites were oestrone- and

oestradiol-3-glucosiduronates in addition to smaller amounts of other phenolic steroid metabolites conjugated with glucuronic acid. There was no obvious excretion of monoglucoside or double conjugate at these later times.

It is abundantly clear from these results that the metabolism of the 3- and 17-monoglucosides of oestradiol are quite different from each other in the human. It seems possible that, in view of the excretion curve for ^{3}H following injection of ^{3}H-oestradiol-17-glucoside, the double conjugate formed, namely oestradiol-3-glucosiduronate-17-glucoside, could be largely excreted via the bile with subsequent reabsorption and production of the 3-glucosiduronates of oestradiol and oestrone as major urinary metabolites; i.e., in a similar fashion as for the enteric metabolism of oestradiol-3-sulphate-17-glucosiduronate. There is little evidence to suggest the formation of free oestradiol from intravenously injected oestradiol-17-glucoside at sites other than enteric ones. This is in marked distinction to the picture for oestradiol-3-glucoside in which extensive hydrolysis occurred giving rise to free oestradiol and hence to major amounts of more highly oxygenated steroidal metabolites.

The metabolism of intravenously injected ^{3}H-17α-oestradiol-6,7-^{3}H-17-β-D-glucopyranoside in normal women (Williamson, Layne, Nilsen and Hobkirk, Can J Biochem 50: 958, 1972) was shown to be somewhat similar to that of 17β-oestradiol-17-glucoside. Thus, a small amount of unchanged glucoside was excreted at an early time together with a labelled double conjugate identified

with some certainty as 17α-oestradiol-3-glucosiduronate-17-glucoside. However, the main, and almost sole, monoglucosiduronate excreted was 17α-oestradiol-17-glucosiduronate. This latter increased with time as the excretion of the monoglucoside and mixed conjugate virtually stopped. A very small amount of a compound tentatively identified as 17α-oestradiol-3-glucosiduronate appeared in the urine beyond 6 hr. The route and site of synthesis of 17α-oestradiol-17-glucosiduronate from the injected 17-monoglucoside is not at present clear.

General Discussion and Comparison of Metabolism of Oestrogen Glucosiduronates and Glucosides

There is now adequate information, provided in the present thesis, as to the great differences in metabolism of oestrogen glucosiduronates on the one hand and glucosides on the other, in the human subject. The obvious reason for this is the presence or absence of the ionizable carboxyl group on the carbohydrate moiety. This in turn is probably related to the ability of the uncharged glucoside conjugate to penetrate cells and/or to the distribution of β-glucuronidase and β-glucosidase enzymes. The 3-glucoside of 17β-oestradiol is metabolized largely by hydrolysis to free 17β-oestradiol (Hobkirk, Nilsen, Williamson and Layne, J Clin Endocr 32: 476, 1971) whereas the 3-glucosiduronate of 17β-oestradiol undergoes very little hydrolysis indeed (Hobkirk and Nilsen, Steroids 15: 649, 1970). 17β-Oestradiol-17-glucosiduronate undergoes metabolism largely through production of 17β-oestradiol-3-sulphate-17-

glucosiduronate which is excreted in the bile and reabsorbed, with deconjugation and reconjugation, to be excreted in the urine as the 3-glucosiduronates of oestrone and oestradiol (see above). The 17-glucosides of 17β-oestradiol and 17α-oestradiol may be metabolized in a somewhat similar fashion to 17β-oestradiol-17-glucosiduronate in that a double conjugate, in these instances the 3-glucosiduronate-17-glucoside, is formed as an intermediate in glucosiduronate production. However, there is as yet no absolute evidence that the latter double conjugate is excreted via the bile.

A knowledge of the metabolism of such conjugates may be of importance in a better understanding of the distribution and metabolism of certain steroidal preparations (including their conjugates) which are used in clinical medicine. Moreover, it is intriguing to enquire into the relationship between oestrogens and mucopolysaccharide synthesis and metabolism especially in view of the interesting finding of 17α-oestradiol specifically conjugated with both N-acetylglucosamine and glucuronic acid in the rabbit (119). A recent review of the subject by Layne (122) includes such points of interest.

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APPENDIX

PUBLICATIONS

SECTION A

ASPECTS OF CONJUGATE HYDROLYSIS AND STEROID METHODOLOGY

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HYDROLYSIS OF ESTROGEN CONJUGATES IN DIABETIC PREGNANCY URINES*

To the Editor:

The most acceptable conditions for the acid hydrolysis of urinary estrogen conjugates, namely, boiling the urine with 15 volumes per cent of concentrated hydrochloric acid for sixty minutes under reflux (1), have been incorporated into 2 reliable procedures for measuring urinary estriol, estrone and estradiol-17β (2, 3). Even under these circumstances, destruction of estrogen still occurs, but it may be minimized by diluting the urine with water prior to hydrolysis (4).

One substance reported to increase destruction of estrogen during acid hydrolysis is glucose (5). In this laboratory, preliminary findings in a study of estrogens in diabetic pregnancy indicated that estriol, estrone and estradiol excretion was markedly low in a number of cases. Because of the difficulties involved in acid hydrolysis and the possible effect of urinary glucose on recovery of estrogen from urine, we carried out the investigation reported here.

MATERIALS AND METHODS

Complete 24-hour urine specimens were collected and stored at 5° C. or in a deep-freeze until analyzed. Under these conditions there was no loss of estriol, estrone or estradiol during the storage period. Determinations of sugar were semiquantitative, using Clinitest tablets (Ames Co. of Canada, Ltd., Toronto).

Acid hydrolysis was carried out on 100-ml. samples of undiluted urine, by the method of Bauld (3). For diluted urine (mid or late pregnancy), samples containing 50, 25 or 10 ml. were diluted to 100 ml. with water to yield dilutions of 1:2, 1:4 and 1:10 respectively, prior to acid hydrolysis. For a 1:20 dilution, a 25-ml. aliquot of urine was diluted to 500 ml. For early pregnancy or other urines with low estrogen levels, a 50-ml. aliquot was diluted to 500 ml. to yield a 1:10 dilution.

For enzyme hydrolysis, a 10-ml. or 20-ml. volume of mid or late pregnancy urine was adjusted to pH 6.5 (glass electrode) with dilute sodium hydroxide or acetic acid.

* Supported by grants from Ayerst, McKenna & Harrison Limited; The Banting Research Foundation; Parke, Davis and Company; and the Women's Auxiliary of The Montreal General Hospital.

TABLE 1. ESTROGEN LEVELS AS MEASURED IN DIABETIC PREGNANCY URINES USING ACID HYDROLYSIS AFTER DIFFERENT DEGREES OF DILUTION OF THE URINE

(Results expressed as μg. per 24 hours)

Urine specimen	Estrogens	Degree of dilution of urine			
		1:2	1:4	1:10	1:20
M_1*	estriol	4,840		9,610	
	estrone	89		119	
	estradiol	47		122	
M_2*†	estriol	5,466		9,923	
	estrone	194		306	
	estradiol	84		196	
M_3*	estriol		9,600	8,020	
	estrone		114	116	
	estradiol		106	87	
L_1	estriol		4,160	5,760	6,360
(½% sugar)	estrone		95	132	92
	estradiol		60	90	104
B_1	estriol		9,920	12,300	
(½% sugar)	estrone		774	858	
	estradiol		124	136	

* Sugar content not measured.

† Premarin (estrogen conjugate preparation—Ayerst, McKenna & Harrison, Montreal) in a dosage of 15 mg. daily was administered to the patient at this time.

This was incubated at 37° C. for twenty-four hours with 20 units of bacterial β-glucuronidase[1] per ml. of original urine. After cooling, the solution was diluted to 100 ml. for extraction. The conditions of incubation were chosen on the basis of results obtained in comparative experiments (unpublished), in which acid and enzyme hydrolysis were employed.

The chemical determination of estriol, estrone and estradiol was made in duplicate by a modification of Bauld's method (3). This involved omitting saponification of the estrone and estradiol fractions after chromatography, and dispensed with chromatography of the estriol fraction after saponification. This is permissible when estrogen levels are high. Average recoveries of estriol, estrone and estradiol added to normal urine after acid hydrolysis are 80, 90 and 90 per cent respectively, by this procedure.

RESULTS

Table 1 shows striking differences in estrogen levels between 1:2 and

[1] Glucuronidase of bacterial origin (Sigma Chemical Co., St. Louis, Missouri). The activity is expressed in Sigma units as defined by the manufacturers.

TABLE 2. COMPARISON OF ACID AND ENZYME HYDROLYSIS OF ESTROGEN CONJUGATES IN DIABETIC PREGNANCY URINES

(Results expressed as μg. per 24 hours)

Urine specimen	Estrogen	Acid hydrolysis		Enzyme hydrolysis
		No dilution	1:10 dilution	
M_4* (2% sugar)	estriol	2,310	11,440	11,000
	estrone	264	986	990
	estradiol	33	360	646
M_5† (2% sugar)	estriol	3,480	16,278	14,920
	estrone	195	267	279
	estradiol	40	157	159
L_2 (2% sugar)	estriol	5,750	15,900	16,750
	estrone	113	142	163
	estradiol	100	144	125
L_3 (no sugar)	estriol	14,000	16,459	17,000
	estrone	175	166	163
	estradiol	150	148	125
		(1:2.5)‡	(1:25)	
S (no sugar)	estriol	15,350	22,115	17,750
	estrone	300	470	318
	estradiol	216	363	320

* Patient received 15 mg. of Premarin per day (see Table 1).

† Patient received 175 mg. of stilbestrol per day.

‡ Urine S was diluted to 2500 ml. before acid hydrolysis; this resulted in a dilution of 1:2.5.

1:10 dilution (urines M_1 and M_2) but no such variation between 1:4 and 1:10 for another urine, M_3. In 2 other urines (L_1 and B_1) there were apparent differences between 1:4 and 1:10 dilutions. Also, an increase in estriol was observed when urine L_1 was diluted from 1:10 to 1:20, although estrone and estradiol values become inaccurate at the higher dilution. The very large differences which may occur in estrogen levels between undiluted urines, and urines diluted 1:10 before acid hydrolysis, are particularly obvious in Table 2 (urines M_4, M_5 and L_2). The much smaller effect of dilution for urine L_3 (no sugar) suggests the relationship of urinary sugar to destruction of estrogen. Although in urine S, with no sugar, there was an increase in all 3 estrogens upon dilution, it should be noted that the lower dilution in this case was 1:2.5 and the higher, 1:25.0. In general, estrogen

TABLE 3. EFFECT OF ADDED GLUCOSE ON THE ACID HYDROLYSIS OF URINARY ESTROGEN CONJUGATES IN NORMAL AND DIABETIC PREGNANCY URINES

(Results expressed in μg. per 24 hours)

Urine specimen*	Degree of dilution	Estriol	Estrone	Estradiol
S	1:2.5	15,350	300	300
S	1:25	22,115	470	363
S+5% glucose	1.2.5	6,610	140	87
S+5% glucose	1:25	17,600	323	198
H	1:10	8,963	1,792	387
H+5% glucose	Nil	1,353	401	44
H+5% glucose	1:10	7,255	1,444	333

* S = diabetic pregnancy urine containing no sugar.
H = normal pregnancy urine.

excretion values after enzyme hydrolysis showed good correlation with those after acid hydrolysis of the 1:10 diluted urines M_4, M_5, L_2 and L_3 (Table 2). For urine S (1:25 dilution) lower values were found after enzyme hydrolysis than after acid hydrolysis.

Table 3 shows that the addition of 5 per cent glucose to pregnancy urine prior to acid hydrolysis resulted in loss of estrogen. Also, this could be largely, if not wholly, compensated for by a tenfold dilution with water prior to hydrolysis.

DISCUSSION

It is apparent that dilution of diabetic pregnancy urine before acid hydrolysis results in a general increase in values for estriol, estrone and estradiol excretion. The extent of this increase seems to be variable for the 3 estrogens. Thus in 3 urines with similar sugar content (M_4, M_5 and L_2; Table 2), acid hydrolysis after 1:10 dilution resulted in an increase varying from 30 per cent (estrone in L_2) to 1100 per cent (estradiol in M_4) over that for undiluted urine. In a sugar-free urine (L_3) there was only a modest increase of estriol (15 per cent) on dilution. This is the expected observation when normal (nondiabetic) urine is diluted 1:10 before acid hydrolysis (4). The greatest destruction of estrogen occurs when acid hydrolysis is performed in undiluted urines containing glucose, and it is evident that urinary estrogen values based on these methods are open to considerable criticism.

The good agreement between estrogen values measured after acid hydrolysis in a 1:10 dilution of urine and those after enzyme hydrolysis, although not constituting proof for completeness of estrogen release, suggests that either procedure is capable of yielding more meaningful results than

those obtained following hot-acid treatment of the undiluted urine. One interesting feature (Table 2) is the significantly lower estriol and estrone levels observed after enzyme hydrolysis than after acid hydrolysis of a 1:25 dilution of urine S. One possible explanation is the presence of estrogen conjugates, other than glucuronides, not hydrolyzed by β-glucuronidase and possibly destroyed during acid hydrolysis at lower dilutions of urine.

It is concluded that any investigation of urinary estrogens (estriol, estrone and estradiol) in diabetic pregnancy requires that hydrolysis of the conjugates be effected either by acid hydrolysis following 1:10 dilution of the urine or by incubation with β-glucuronidase. There can be little doubt that glucose is a major factor in loss of estrogen during acid hydrolysis of undiluted diabetic urines. This suggestion receives considerable support from the data in Table 3.

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HYDROLYSIS OF CONJUGATED ESTROGEN FRACTIONS IN HUMAN PREGNANCY URINE[1]

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Abstract

The release of six estrogen fractions from conjugation in human pregnancy urines has been studied using various hydrolytic methods. The estrogens concerned were estrone, estradiol-17β (estradiol), 2-methoxyestrone, 16-epiestriol, and a ring D ketolic fraction (mainly 16α-hydroxyestrone). Considerable amounts of urinary estrone and ring D ketolic estrogens may be conjugated in a non-glucuronide form. In these cases an enzyme preparation containing β-glucuronidase and sulphatase, prepared from the digestive juice of the snail *Helix pomatia*, proved to be superior to β-glucuronidase enzymes of bacterial or mammalian liver origin. Conventional hot acid hydrolysis yielded levels of estrone, estradiol, estriol, and 16-epiestriol which agreed fairly well with those obtained following snail enzyme hydrolysis. In some urines, hot acid treatment was not suitable for hydrolysis of conjugated 2-methoxyestrone. Optimum hydrolytic conditions for both normal and diabetic pregnancy urines were realized by incubating for 24 hours with 500 units of the snail β-glucuronidase and 250 units of sulphatase/ml of urine at pH 5.2 and 37–38° C.

Introduction

The problem of hydrolyzing urinary estrogen conjugates prior to estrogen analysis is not a new one. Within recent years, however, new problems have arisen due to the discovery of labile ring D ketolic compounds (e.g. 16α-hydroxyestrone) which cannot be treated by conventional hot acid hydrolysis without destruction (1). Moreover, in urines containing glucose, destruction of even the relatively stable 'classical' estrogens, namely, estrone, estradiol-17β (estradiol), and estriol, has been observed during hot acid treatment (2, 3). Since the ring D ketolic estrogens seem to be of considerable importance in the general scheme of estrogen metabolism (4), and since estrogen patterns in diabetic pregnancy urines may be of interest (5), mild methods of hydrolysis, such as incubation with β-glucuronidase and sulphatase preparations, have become increasingly necessary.

A number of investigators have reported upon the efficiency of enzyme hydrolysis as compared with conventional hot acid treatment (2, 6–10). Such studies have also yielded some indirect information regarding the mode of conjugation of certain of the urinary estrogens. However, little is known about the hydrolytic release of the more recently discovered metabolites such as 2-methoxyestrone, 16-epiestriol, and 16α-hydroxyestrone. Knowledge of this sort might lead to a clearer understanding of the over-all metabolism of estrogen secreted by the human subject.

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The present report concerns the use of various hydrolytic techniques for the cleavage of conjugated estrogen metabolites in human pregnancy urine. The compounds investigated include estrone, estradiol, estriol, 2-methoxyestrone, 16-epiestriol, and a fraction referred to as 'ring D ketolic', this latter apparently consisting mainly of 16α-hydroxyestrone but also containing 16-ketoestradiol-17β and probably 16β-hydroxyestrone. Particular attention has been given, in the present study, to various aspects of the action of an enzyme preparation from the digestive juice of the snail *Helix pomatia*.

Material and Methods

Urine Collection

Complete 24-hour specimens were collected without preservative from some 25 women, both normal and diabetic, during the last 20 weeks of pregnancy. Following the final collection each urine was despatched to the laboratory without delay and hydrolysis was immediately begun. Where delays were necessary between the termination of enzyme incubation and subsequent analysis the urine was frozen after incubation until required.

Chemicals

Organic solvents and other reagents were Reagent Grade and were purified where necessary by published procedures (11, 12).

Enzyme Preparations

Bacterial β-glucuronidase, Sigma Chemical Co.; mammalian liver β-glucuronidase (Ketodase), Warner Chilcott Laboratories Inc.; molluscan β-glucuronidase plus sulphatase (Glusulase), prepared from the snail *Helix pomatia*, Endo Laboratories Inc.; molluscan β-glucuronidase plus sulphatase, prepared from the common limpet *Patella vulgata;* powder B of Dodgson and Spencer (13).

The unit of activity of each preparation was based on its β-glucuronidase content and is defined as the amount of enzyme liberating 1 μg of phenolphthalein from a 0.001 *M* solution of phenolphthalein glucuronide in 1 hour at 37° C.* The pH of incubation was 6.5 for the bacterial preparation, 5.0 for the liver enzyme, 5.2 for the snail preparation, and 4.6 for the limpet material.

Hydrolysis of the Estrogen Conjugates

For enzyme hydrolysis, 10- or 20-ml volumes of urine were incubated, in duplicate, with one or the other of the enzyme preparations at varying enzyme concentrations, pH values, or for varying times, at 37–38° C. Adjustment of pH for the various enzymes was made as described earlier (15). In most cases penicillin (2000 units/ml of urine) was added prior to incubation (16).

Where hot acid hydrolysis was performed 10- or 20-ml volumes of urine, diluted 1:10 with distilled water, were hydrolyzed by boiling for 1 hour under

*The unit of activity of the bacterial preparation should be modified by indicating that assay was performed in the presence of chloroform. This exerts an 'activating' effect (14) and the unit is less than is the case in the absence of chloroform when phenolphthalein glucuronide is the substrate.

reflux with 15 volumes % of HCl (11); this acid concentration referring to diluted urine.

Splitting of residual labile estrogen conjugates (e.g. sulphates) following enzyme hydrolysis was performed by adjusting the urine (from which free estrogens had already been extracted) to pH 1 with HCl and continuously extracting with ether in an all-glass apparatus for 48 hours at room temperature (17). Estrogen thus released was considered to have arisen from labile non-glucuronide conjugates which were not hydrolyzed by the enzyme preparations.

Extraction and Analysis of the Estrogens

In all cases incubated urine was diluted to 100 ml with water prior to extraction. In experiments where only estrone, estradiol, and estriol were to be measured, hydrolysis was followed by the modified method of Bauld (3, 18). For the analysis of six estrogen fractions, including the labile ring D ketolic compounds, the method of Givner *et al.* (12) was employed, modified by omitting the second Girard separation and saponification of the estradiol and 2-methoxyestrone fractions following partition chromatography, and by reducing solvent and reagent volumes where necessary. Where the ring D ketolic fraction was not to be measured, as in analyses following hot acid hydrolysis, the initial NaOH–bicarbonate treatment of the ether extract was employed according to Bauld (11), followed by the Girard separation and subsequent steps of the method of Givner *et al.* (12).

Recovery Experiments

For the purpose of checking the accuracy of the analytical methods, known amounts of pure estrogens (5–25 μg depending on the compound concerned) were added to 10- or 20-ml volumes of non-pregnancy (male or female) urine which had been hydrolyzed either by the enzymic or hot acid method. These urines were then analyzed by the techniques described above. Recoveries were also measured of estrogens (excluding the ring D ketolic compounds) added to urine prior to hot acid hydrolysis. In studying the recovery of the ring D ketolic estrogens 16α-hydroxyestrone was normally employed as the representative compound but some experiments were also carried out using 16-keto-estradiol-17β.

Results

Accuracy of the Methods

Table I deals with the accuracy of the analytical procedures. Recovery of the various estrogens was not influenced by the enzyme preparation used. With the possible exception of 16-epiestriol, recovery of the pure estrogens was also independent of whether these were added to enzyme- or acid-hydrolyzed urine. Recovery of added estrone, estradiol, estriol, and 2-methoxyestrone was the same whether these steroids were added before or after hot acid hydrolysis. Good agreement, considering the different techniques involved, existed between the methods of Bauld (11) and Givner *et al.* (12) with respect to recovery of estrone, estradiol, and estriol.

TABLE I

Percentage recoveries of estrogens added to non-pregnancy urines
(Mean values are given ± standard deviation or with ranges in parentheses)

Analytical method	Hydrolytic method	Estrogen added					
		Estrone	Estradiol	Estriol	2-Methoxy-estrone	16-epiEstriol	16α-Hydroxyestrone or 16-ketoestradiol-17β
Givner *et al.* (12)	Enzyme*	85 ± 3.7 *n* = 22§	80 ± 5.6 *n* = 14	75 ± 5.0 *n* = 23	72 ± 6.0 *n* = 12	75 ± 5.8 *n* = 14	64 ± 4.7 *n* = 16
Givner *et al.* (12)	Hot acid†	90(85–96) *n* = 4	81(79–85) *n* = 4	71(64–75) *n* = 4	76(74–81) *n* = 4	84(76–90) *n* = 4	—
Givner *et al.* (12)	Hot acid‡	86(83–87) *n* = 3	79(77–83) *n* = 3	72(67–78) *n* = 3	77(70–88) *n* = 3	78(75–83) *n* = 3	—
Bauld (11)	Hot acid‡	92 ± 2.1 *n* = 10	83 ± 2.5 *n* = 10	80 ± 3.2 *n* = 12	—	—	—

*Employing 20 units bacterial enzyme/ml at pH 6.5; 300 units liver enzyme, pH 5.0; 500 units snail preparation, pH 5.2; *or* 750 units limpet preparation, pH 4.6.
†Estrogens added after hydrolysis.
‡Estrogens added before hydrolysis.
§*n* = number of duplicate analyses.

Hydrolysis by Various Methods

The effect of varying incubation time on the enzymic release of the three main estrogen fractions, estriol, estrone, and ring D ketolic estrogens, is shown in Figs. 1, 2, and 3. Within 48 hours of incubation the bacterial, limpet, and

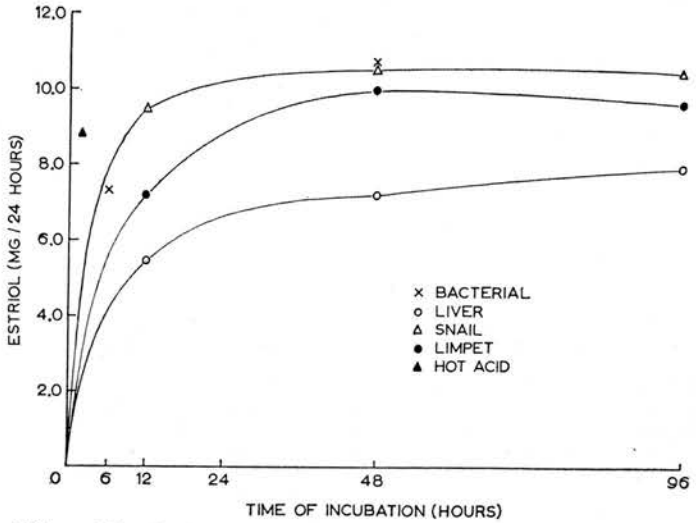

FIG. 1. Effect of incubation time on the liberation of estriol in normal human pregnancy urine by β-glucuronidase-containing preparations of bacterial, mammalian liver, and molluscan origin. Conditions of hydrolysis as in Table I and in text.

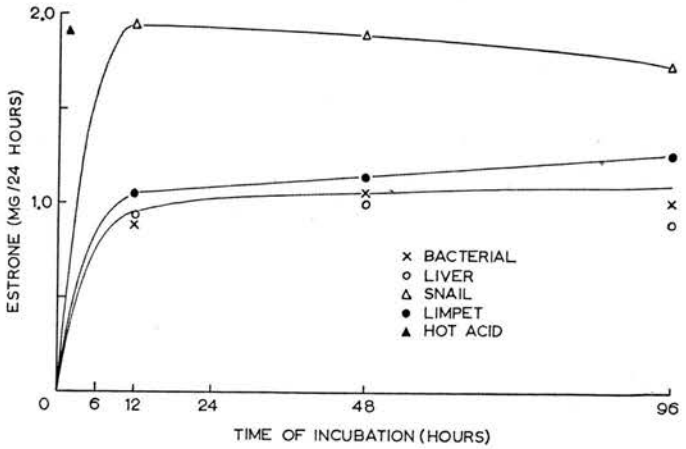

FIG. 2. Effect of incubation time on the liberation of estrone in normal human pregnancy urine by β-glucuronidase-containing preparations of bacterial, mammalian liver, and molluscan origin. Conditions of hydrolysis as in Table I and in text.

snail preparations gave rise to rather higher estriol levels than did conventional hot acid hydrolysis. Continuous ether extraction following 96 hours of snail enzyme activity released only an additional 3% of estriol while a further 2%,

4%, and 10% was released by this procedure after incubation with bacterial (48 hours), limpet (96 hours), and mammalian liver (96 hours) enzymes, respectively. It was apparent from this that little estriol was present as a labile conjugate. The difference between the liver enzyme and the other preparations was considered to be due to slower hydrolysis of estriol glucuronide by the former enzyme (19).

In a second normal pregnancy urine, maximum hydrolysis of estrone conjugates was attained within 12 hours with all four enzyme preparations (Fig. 2). However, this maximum was much greater in the case of the snail preparation, the value being the same as that obtained by hot acid hydrolysis. Continuous ether extraction following incubation with the other three enzyme preparations released sufficient estrone to account for the observed differences, suggesting considerable (40–50%) conjugation of the steroid in a non-glucuronide form in this particular urine and perhaps even a labile conjugate, other than sulphate, split by the snail preparation but not by the sulphatase-containing limpet one. Very similar results were obtained for the ring D ketolic fraction in the same urine (Fig. 3). Continuous ether extraction subsequent to enzyme hydrolysis resulted in no further measurable release of ring D ketolic estrogens,

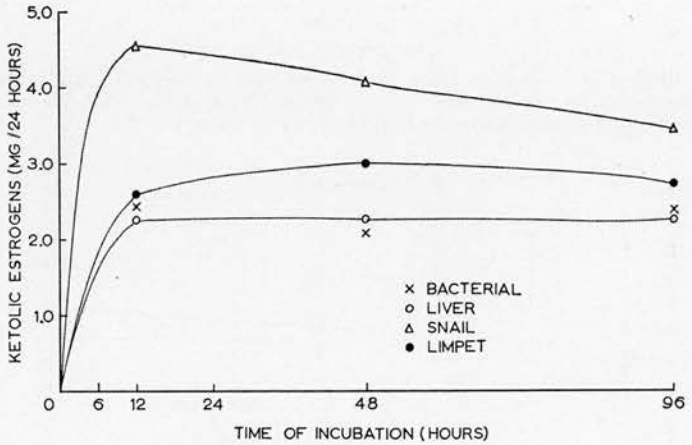

FIG. 3. Effect of incubation time on the liberation of ketolic estrogens in normal human pregnancy urine by β-glucuronidase-containing preparations of bacterial, mammalian liver, and molluscan origin. Conditions of hydrolysis as in Table I and in text.

presumably because of the labile nature of these compounds under acidic conditions. It can be seen in Fig. 3 that a marked decrease occurred in the ring D ketolic fraction when incubated with the snail preparation between 12 and 96 hours. This was also true of the estrone fraction in the same urine (Fig. 2). Antibiotics were not used in these particular incubations. Even in the presence of penicillin, however, a similar fall occasionally occurred in the ring D ketolic fraction.

Table II compares the efficiencies of different hydrolytic methods for the

TABLE II

Liberation of estrone (O), estradiol (D), and estriol (T) in a normal human pregnancy urine by different hydrolytic methods (Results expressed as mg/24 hours)

Hydrolytic* method	O + D + T liberated	O + D + T liberated by continuous extraction after enzyme hydrolysis	Total	Total, as % of snail enzyme + continuous extraction
Hot acid	11.6	—	11.6	87
Bacterial enzyme	12.7	0.73	13.4	101
Mammalian liver enzyme	10.0	1.33	11.3	85
Limpet enzyme	11.8	0.59	12.4	93
Snail enzyme	13.0	0.34	13.3	100

*Enzyme concentrations and conditions of pH as in Table I; times of hydrolysis were bacterial, 48 hours; mammalian liver, 96 hours; snail, 96 hours; and limpet, 96 hours.

release of estrone, estradiol, and estriol in a normal pregnancy urine. All four enzyme preparations used yielded results which compared at least favorably with those obtained by hot acid hydrolysis. When five normal pregnancy urines were separately hydrolyzed with hot acid, snail preparation, and bacterial preparation, and also analyzed for estrone, the results (Table III) suggested very variable amounts of this steroid to be present as non-glucuronide ('sulphate') conjugates.

TABLE III

Liberation of estrone in normal human pregnancy urines by different hydrolytic methods (Results expressed as mg/24 hours)

Hydrolytic* method	Normal pregnancy urines from different subjects				
	1†	2†	3	4	5
Hot acid	2.4	1.9	0.59	0.69	0.77
Snail enzyme	2.4	1.9	0.62	0.69	0.76
Bacterial enzyme	1.6	1.1	0.55	0.65	0.59
Bacterial, as % of hot acid or snail enzyme	67	58	90	94	77

*Enzyme concentrations and conditions of pH as in Table I; time of hydrolysis for enzymes, 24 hours.
†Estrone liberated by continuous ether extraction following action of bacterial enzyme accounted for difference between the latter and the other hydrolytic methods.

Effect of pH on the Activity of the Snail Preparation

Aliquots of a normal pregnancy urine were incubated for 24 hours with amounts of the snail preparation corresponding to 500 units of β-glucuronidase activity/ml of urine at pH values of 4.0–6.5 at pH unit intervals of 0.5. Release of the six estrogen fractions measured did not vary significantly over the pH range studied except in the case of estriol which showed a considerable decrease at 6.5 (70% of the level obtained between pH 4.0 and pH 6.0).

Effect of Variation of the Snail Enzyme Concentration

Aliquots of five urines were incubated with varying concentrations of the snail preparation (100–1000 units of β-glucuronidase/ml of urine). Table IV contains the results obtained. In only three of the urines was measurable 2-methoxyestrone found (0.19–0.66 mg/24 hours). It was observed that 100 units of activity was sufficient for maximum hydrolysis of conjugated estrone, estradiol, 16-epiestriol, and the ring D ketolic fraction in 24 hours, but not for 2-methoxyestrone or estriol. Five hundred units appeared to offer a safe compromise since 1000 units did not result in any consistent increase in hydrolysis.

Effect of Variation of Incubation Time on Activity of the Snail Preparation

Aliquots of five pregnancy urines were incubated with the snail enzyme preparation for varying times from 6 to 96 hours. In only two of these was measurable 2-methoxyestrone found (0.19–0.30 mg/24 hours). The results in Table V show considerable hydrolysis to have occurred in 6 hours for all fractions, with 2-methoxyestrone and estriol lower than the others. In 16 hours approximately, maximum release of all fractions was apparent although

TABLE IV

Liberation of six estrogen fractions in human pregnancy urines* by different concentrations of snail enzyme preparation (Mean results (with ranges) expressed as percentage of values obtained with 500 units of enzyme/ml for each urine)

Estrogen fraction	Units of β-glucuronidase/ml of urine				
	100	250	500	750	1000
Estrone	104	100 (94–105)	100	101 (100–103)	99 (98–100)
Estradiol	92	97 (87–100)	100	91 (87–104)	84 (72–96)
Estriol	77	95 (87–101)	100	101 (91–110)	104 (103–105)
2-Methoxyestrone	81	89 (82–99)	100	97 (90–100)	83
16-epiEstriol	108	101 (96–108)	100	97 (92–101)	96 (92–100)
Ring D ketolic fraction	102	104 (98–112)	100	109 (101–118)	100 (93–102)

*Two normal and three diabetic urines were employed with incubation periods of 24 hours at pH 5.2. In the case of all the fractions at the 100-unit level and or 2-methoxyestrone at the 1000-unit level only one urine was analyzed.

96 hours' incubation did result, in some urines, in a further increase in the ring D ketolic fraction. However, as has already been mentioned, decreased ring D ketolic levels could also occur, even in the presence of antibiotics. For these reasons an incubation period of 24 hours was considered to be most suitable. The rapid release of estriol by this snail preparation (Fig. 4) was particularly noteworthy in view of the slower hydrolysis by other enzyme preparations (2, 19).

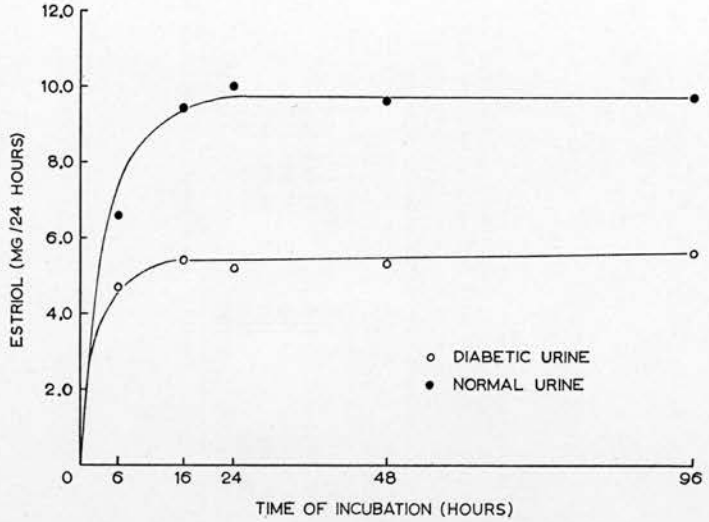

FIG. 4. Effect of incubation time on the liberation of estriol in normal and diabetic pregnancy urines by the snail enzyme preparation. Conditions of hydrolysis as in Table I for the snail preparation.

Comparison of Hot Acid with Snail Enzyme as Hydrolytic Methods

Aliquots of pregnancy urines (both normal and diabetic) were incubated for 24 hours at 37° C and pH 5.2 with a concentration of the snail preparation equivalent to 500 units of β-glucuronidase/ml of urine. Five estrogen fractions (excluding the ring D ketolic estrogens) were measured, and their amounts compared with those liberated in the same urines by hot acid hydrolysis after 1:10 dilution. The results for four fractions are shown in Table VI. Good agreement was found between the two hydrolytic methods for the release of estrone, estradiol, and estriol with the results slightly, although not significantly, in favor of enzyme hydrolysis. The converse was true for 16-epiestriol although again there was no significant difference. These findings are in agreement with those of other workers who used limpet enzyme preparations (2, 20).

In four urines the 2-methoxyestrone released by acid amounted to 60–87% (mean, 77%) of that obtained by snail enzyme hydrolysis. In two other urines no measurable 2-methoxyestrone was found after hot acid hydrolysis although 0.2 and 0.3 mg of this steroid/24 hours was measured after incubation with the snail preparation. These results are difficult to interpret since preliminary

TABLE V

Liberation of six estrogen fractions in human pregnancy urines* when incubated for varying times with the snail enzyme preparation (Mean results (with ranges) expressed as percentages of values obtained after 24 hours for each urine)

Estrogen fraction	Time of hydrolysis (hours)				
	6	16	24	48	96
Estrone	95 (89–103)	98 (93–102)	100	98 (93–105)	95 (89–104)
Estradiol	92 (91–93)	99 (88–109)	100	96 (91–104)	111 (109–112)
Estriol	78 (66–92)	97 (92–104)	100	101 (96–107)	103 (97–109)
2-Methoxyestrone	75	100 (97–103)	100	96 (82–109)	97 (82–111)
16-epiEstriol	99 (92–103)	103 (97–106)	100	99 (94–100)	104 (102–106)
Ring D ketolic fraction	90 (88–93)	95 (88–100)	100	97 (91–100)	96 (76–115)

*Three normal and two diabetic urines were employed with enzyme concentrations of 500 units/ml of urine at pH 5.2. In the case of 2-methoxyestrone at 6-hour incubation time only one urine was analyzed.

TABLE VI

Comparison of hot acid and snail enzyme hydrolysis
(Results expressed as acid/enzyme × 100; mean values are given with number of duplicate analyses in parentheses)

Estrone	Estradiol	Estriol	16-epiEstriol	Total*
95 ± 7.6† (15)	89 ± 17.0† (9)	89 ± 6.5† (9)	109 ± 16.7† (10)	90 ± 6.7† (8)

*Total refers to the addition of the four estrogen fractions in the table.
†Standard deviation.

experiments in this laboratory showed free 2-methoxyestrone to be stable to hot acid hydrolysis in the presence of urine. In some urines, however, considerable 'background' chromogen contamination was produced in the 2-methoxyestrone fraction by acid hydrolysis. This could be particularly troublesome when very low levels of this steroid were being measured. This observation, together with the possibility that the effect of acid on free and conjugated 2-methoxyestrone may not be the same, could perhaps account for the above results.

Discussion

Since recovery of the various estrogens added to hydrolyzed urine was independent of the hydrolytic method employed, the difference in estrogen levels found after incubation of certain urines with enzyme preparations from different sources was probably a true reflection of a variation in the ability of these enzymes to split urinary estrogen conjugates under the experimental conditions. The amounts of bacterial, mammalian, and limpet enzymes used in the present study, although not necessarily ideal, have been shown by others to yield optimum hydrolysis of estrogen conjugates given sufficient time for action to occur (2, 21, 22). It is improbable that higher concentrations of bacterial or mammalian preparations could have increased the extent of hydrolysis of conjugated estrone in those urines where the snail preparation released significantly more of this steroid, since the difference was due to labile (non-glucuronide) conjugates. There is no good evidence that enzymes capable of splitting the latter are to be found in either the bacterial or mammalian liver preparations. In the case of the limpet preparation, however, one would have expected to observe a greater release of estrone provided that the non-glucuronide conjugate was sulphate. This is discussed further below. These observations, together with the facility possessed by the snail preparation for releasing estriol and ring D ketolic estrogen metabolites, showed that this enzyme mixture was a very suitable one for the present purpose. Slaunwhite and Sandberg (23) have reported the snail preparation to be better than enzymes of bacterial or mammalian liver origin for the hydrolysis of conjugated estrogens in urine. These workers obtained maximum hydrolysis by incubating urine for 96 hours at pH 5.5 and 37° C with 300 units/ml of the snail β-glucuronidase together with sulphatase. These results were based on the measurement of

total radioactivity released in the urine following the administration of C^{14}-labelled estrogen to human subjects and therefore provided no information regarding the effect of this hydrolytic procedure on individual metabolites.

It was established by Brown and Blair (2) that, provided urine was first diluted 1:10 with water, no destruction of added free estrone, estradiol, or estriol occurred during hot acid hydrolysis. Moreover, this method of hydrolysis was shown to yield levels of these three estrogens which compared very closely with those obtained by incubating with the limpet enzyme preparation. Thus it appeared likely that the limpet enzymes were capable of affecting more or less complete release of the metabolites in question. By the same token it could be suggested from the results of the present study that this is also true of the snail preparation and that it extends to include 16-epiestriol. A few results obtained showed that the snail preparation could be significantly more active than the limpet one in releasing estrone and ring D ketolic estrogens. In the case of estrone this was apparently due to a failure to hydrolyze non-glucuronide conjugates since continuous ether extraction at pH 1 accounted for the difference. This finding was surprising in view of the sulphatase present in the limpet preparation, which enzyme would be expected to split estrone sulphate. It is not clear at present whether a non-glucuronide conjugate, other than sulphate, could have existed in this urine and might have been hydrolyzed by some other enzyme present in the snail preparation but absent from the limpet material. Bloch and Gibree (10) reported much higher (70–100%) levels of estrone, estradiol, and estriol following incubation with 1500 units of snail β-glucuronidase, and at least 500 units of sulphatase/ml of urine for 24 hours at pH 5.2 and 39° C, than after hot acid hydrolysis. However, these workers used non-pregnancy urines of very low estrogen titer without dilution prior to acid hydrolysis; these conditions were known to be quite destructive (2). Jayle *et al.* (24) used 750–1500 units of snail β-glucuronidase and at least 500 units of sulphatase/ml of urine for the hydrolysis of conjugated estrone, estradiol, and estriol and obtained better results than with either bacterial β-glucuronidase or hot acid. In the present study, each 500 units of snail β-glucuronidase was accompanied by approximately 250 units of sulphatase activity.

Comparison of hot acid and enzyme hydrolysis has not been possible for conjugated ring D ketolic estrogens or 2-methoxyestrone due to the adverse effect of hot acid on these fractions. Moreover, the picture is further complicated for 2-methoxyestrone by the number of pregnancy urines in which measurable amounts of this steroid do not seem to occur (25). Thus no definite conclusion has been reached regarding the quantitative release of these estrogens by the snail preparation. However, it does seem likely that the hydrolytic conditions finally chosen are capable of yielding approximately maximum release of the six estrogen fractions concerned. In none of the experiments performed was there any marked difference in hydrolytic efficiency by the snail preparation whether normal or diabetic urines were studied. This showed that

the enzymes were not adversely affected by the usual constituents of diabetic urine.

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URINARY ESTROGENS IN NON-PREGNANT HUMAN SUBJECTS MEASURED BY MODIFICATIONS OF BAULD'S METHOD.

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Abstract

The chemical method of Bauld, and certain modifications of it, for the measurement of urinary estrone, estradiol-17β and estriol may sometimes overestimate these compounds in males and post-menopausal females. A more elaborate procedure, involving a double partition chromatographic treatment together with the Ittrich fluorimetric procedure, yields results which compare more favourably with those obtained by the Brown and other methods. For control male subjects the results obtained are (μg./24 hours); estrone, average = 3.5, range = 0.8 - 7.1; estradiol-17β = 0.9 (0.4 - 1.7); estriol = 6.1 (1.5 - 10.4). In post-menopausal females estrone = 1.2 (0.2 - 3.1); estradiol = 0.4 (0.0 - 0.9); estriol = 4.1 (0.6 - 10.4). The ring D α-ketolic estrogen levels were of the same order as those of estrone. In the males this group measured 3.8 (0.6 - 11.0) and in the post-menopausal females, 1.2 (0.1 - 2.0).

INTRODUCTION

During approximately the last decade the measurement of urinary estrone, estradiol-17β (estradiol) and estriol has been mainly performed by the chemical procedures of Brown' and Brown

et al[2]. These methods have been subjected to a considerable degree of critical evaluation by investigators employing various procedures including bio-assay[3], countercurrent distribution and isotope dilution[4]. Such studies have served to prove the reliability of the Brown methods except for urines of estrogen content lower than the useful sensitivity claimed for the procedures. More recently Salokangas and Bulbrook[5] combined the method of Brown et al with the sensitive and highly specific color reaction of Ittrich[6] and concluded that the former procedure yields an overestimation of the three estrogens which may be particularly significant at low urinary levels. Finkelstein et al[7] have reported that Brown's method grossly overestimates the three estrogens, even at the levels prevailing in the menstrual cycle. However, these latter investigators did not appear to make a direct comparison of their procedure with that of Brown, nor did they publish normal values per 24 hours for their elaborate purification technique.

Few investigators have employed the method of Bauld[8] for estrogen measurement. This is perhaps mainly due to the more elaborate partition column systems which require some degree of temperature control. So far as the present authors are aware the results of only one direct comparison of estrogen levels by the methods of Bauld and Brown have been reported[9]. This related to urines of the menstrual cycle and there was promising agreement

between the procedures. However, indirect evidence, in the form of an examination of published results, may suggest that in male or post-menopausal female urine the Bauld method, or modifications of it, overestimates the levels of estradiol and estriol in particular.[10,11]

In order to explore some of the uncertainties associated with the Bauld procedure, the technique has been combined with that of Givner et al[12] and also with the Ittrich spectrophotometric and fluorimetric methods.

EXPERIMENTAL

Urine Collection

Complete 24 hour urines were collected from hospitalized patients having no evidence of liver or endocrine disease, and from some laboratory personnel. Urines were analyzed as soon after collection as possible. Where delays of more than four days were necessary the material was frozen until required.

Materials

All chemical reagents and organic solvents were of good reagent grade and were purified, where necessary, by established methods. For fluorimetry a Farrand Model A fluorometer was employed with an interference filter (546 mμ) in the primary position and an orange glass filter (max. transmission 585 mμ) in the secondary position. For spectrophotometry a Beckman Model B instrument was used.

Labelled Compounds

Estrone-16-^{14}C (specific activity 40 μc/mg.) was obtained from Radiochemical Centre, Amersham, England. Estriol-16-^{14}C (specific activity 22 μc/mg.) and 16-ketoestradiol-17β-16^{14}C (specific activity 20 μc/mg.) were obtained from Dr. M. Levitz, New York. Estradiol-17β-6, 7-^{3}H (specific activity 150 μc/μg.) was obtained from New England Nuclear Corp., Boston, Mass. All of these were chromatographed in partition systems on celite and/or paper to obtain a purity of at least 98%.

Method A

One-fiftieth aliquots of 24 hour urine specimens were diluted to 50 ml. with water and incubated with 20 units/ml. of bacterial β-glucuronidase (Sigma Chemical Co., St. Louis, Missouri) at 37°C and pH 6.5 - 7.0 for 24 hours. The ether extract of this was then purified as described by Bauld[8] using proportionately less sodium hydroxide, sodium bicarbonate and water for washing. The benzene/water partition and subsequent steps, including column partition for the separation and purification of estrone, estradiol and estriol, were also performed exactly as described by the latter author. One-third aliquots of the column eluates were taken for fluorimetric measurement by the Ittrich technique[6] using tetrabromoethane as solvent. Each compound was measured in terms of the pure material as standard.

One-fiftieth urine aliquots were incubated as above and the

ether extracts washed with sodium bicarbonate and water. A modified Girard separation[13] was then performed and the ketonic fraction was chromatographed on celite in the system benzene/hexane/methanol/water (5:5:7:3) collecting a fraction with the mobility of the ring D α-ketols[11]. The Ittrich fluorimetric reaction was performed on one-third aliquots of these eluates, results being calculated in terms of 16-ketoestradiol-17β.

Method B

Aliquots ranging from 50% to 90% of 24 hour urines were incubated and extracted as in method A using proportionately greater volumes of ether. The ether extracts were washed with sodium bicarbonate and water and subjected to the modified Girard reaction. The non-ketonic fractions were refluxed with alkali, re-extracted after pH adjustment and purified as described by Givner et al[12]. The fractions were then resolved into estrone, estradiol, ring D α-ketols and estriol on celite in hexane/benzene/methanol/water systems as described by the latter authors. The estrone, estradiol and estriol fractions were further purified by Bauld[8]. The ring D α-ketols were reduced with sodium borohydride and the major products resulting, namely, 16-epiestriol and estriol, were separated and further purified by partition on a celite column as described earlier[11 12]. Aliquots of column eluates ranging from one-tenth to one-hundredth were taken for fluorimetric measurement by the Ittrich procedure. Each compound was measured in terms of

the pure material as standard.

Method C

This was exactly the same as method B except that one-third or one-sixth aliquots of the column eluates were taken for spectrophotometric measurement using the Ittrich technique with tetrachloroethane as solvent, reading optical densities at 506, 538 and 570 mμ and applying the Allen correction[14]. Each estrogen fraction was measured in terms of the pure material as standard.

Recovery of Radioactive Estrogens

A few recovery experiments, employing the above methods, were carried out following the addition of small amounts of estrone-16-^{14}C, estriol-16-^{14}C, 16-ketoestradiol-17β-16-^{14}C and estradiol-17β-6,7-^{3}H to urines after enzyme hydrolysis. Recovery of radioactivity was determined on eluate aliquots in a Packard Tricarb Model 314 AX liquid scintillation spectrometer, correcting for quenching where necessary by the use of internal standards. Dried column eluates were dissolved directly in the counting vials in 5 ml. of toluene containing 0.3% of 2,5-triphenyloxazole and 0.01% of 1,4-bis-2-(5-phenyloxazolyl) - benzene except in the case of estriol and 16-epiestriol which were first dissolved in 0.1 ml. of methanol. Counting efficiency for ^{14}C was 73% and for ^{3}H, 21%.

Effect of Column Eluates in Quenching of Fluorescence

Aliquots of column eluates in methods A and B were taken with and without the addition of small amounts (e.g. 0.05 μg.) of the

estrogens in question and treated by the Ittrich fluorimetric procedure. Pure standard estrogens were run concurrently in order to compare the effect of eluates on the fluorescence.

RESULTS

In Table 1 are shown levels of estrone, estradiol and estriol as measured by methods A, B and C in six random urines. It can be seen, as might be expected, that the recovery of radioactivity was somewhat better in method A than in the longer procedures. Also, the overall recovery, including fluorescence quenching effects, was considerably lower than that of the radioactivity alone, indicating that the greater part of the apparent loss in such procedures is due to interference at the fluorimetric stage. For these reasons, the results obtained by methods A and B were corrected for overall losses so that the two procedures could be more closely compared. The results obtained using method C were only corrected on the basis of recovery of radioactivity. In all six estrone fractions a well-defined maximum light absorption was observed at 538 mμ in method C, yielding corrected optical densities > 0.01. In all instances the estrone levels were at or above the lower useful limits of sensitivity of the methods which were for A, B and C, 0.6, 0.1 and 0.4 μg/24 hours respectively with a reagent blank equivalent to 0.002 μg of estrone. Method B and C gave results which agreed well while method A appeared to yield somewhat higher levels. In the case of estradiol the lower useful limits of

sensitivity for methods A, B and C were 1.0, 0.15 and 0.6 μg/24 hours respectively, with a reagent blank equivalent to 0.003 μg of estradiol. For this reason it is difficult to compare the estradiol results in Table 1 since many of them are below the limits of sensitivity. However, urines 4, 5 and 6 suggest an overestimation when using method A. Although the results obtained by methods B and C agreed reasonably well, only the fractions from urines 1 and 4 showed maximum light absorption at 538 mμ in method C. The estriol fractions from all six urines gave well-defined maxima at 538 mμ in method C with corrected optical densities > 0.01. The limits of sensitivity for estriol measurement were about the same as given above for estradiol. The three procedures compared quite favourably with A giving somewhat higher results. In urine 2 method C gave a lower result than did A or B.

Table II contains data on the measurement of ring D α-ketols by methods A, B and C. Although method C yielded in all cases a maximum light absorption at 538 mμ there was considerable background color in some samples. This may partially explain the rather poor agreement between the three methods. Moreover, the recovery figures were disappointingly low. Method A yielded, in general, higher results than did the other two procedures. To what extent this may be due to the composite nature of the fraction measured by method A and to the availability of only one of its components,

TABLE 1

ESTRONE(E_1), ESTRADIOL(E_2) AND ESTRIOL(E_3) CONTENTS OF SIX URINES BY METHODS A, B AND C*

(μg./24 hours)

Subject (age)	E_1			E_2			E_3		
	A	B	C	A	B	C	A	B	C
1. Female (38)	8.9	7.8	7.3	3.9	3.3	2.9	16.0	14.4	13.9
2. Female (64)	0.5	0.3	0.5	0.5	0.1	0.4	3.3	3.4	1.9
3. Female (70)	0.8	0.3	0.5	0.5	0.1	0.0	1.3	1.3	1.5
4. Female (63)	2.3	1.1	1.1	1.0	0.5	0.6	7.7	7.5	7.9
5. Male (19)	4.1	3.7	3.3	3.0	1.1	0.5	2.1	1.7	1.3
6. Male (32)	1.5	0.9	1.0	1.8	0.6	0.7	7.9	6.9	6.7
Mean	3.0	2.4	2.3	1.8	0.9	0.9	6.4	5.9	5.5
Difference (A-B)	0.6			0.9			0.5		
Mean recovery of counts	99	92		91	85		95	86	
Mean overall recovery	80	72		80	68		75	69	

* Results by methods A and B were corrected for difference in overall recovery between these procedures. Results by method C were corrected only for recovery of radioactivity.

TABLE II

RING D α-KETOLS CONTENTS OF FIVE URINES BY METHODS A, B AND C*

(μg./24 hours)

Subject (age)	A	B	C
2. Female (64)	2.1	3.7	5.0
3. Female (70)	4.7	2.0	1.7
4. Female (63)	5.3	4.0	3.2
5. Male (19)	7.0	5.0	2.5
6. Male (32)	7.4	3.4	1.8
Mean	5.1	3.4	2.8
Mean recovery of counts	65	65	
Mean overall recovery	59	56	

* Results by methods A and B were corrected for difference in overall recovery between these procedures. Results by method C were corrected only for recovery of radioactivity in the form of 16-ketoestradiol-17β-16-^{14}C.

namely 16-ketoestradiol-17β-16-^{14}C, for recovery purposes, is uncertain.

In Table III are shown levels of estrone, estradiol and estriol as measured by method B in urines of control male subjects. These results, and those in subsequent tables are means of at least two, and at most four, 24 hour urines. Also shown in Table III, for purposes of comparison are results obtained by other workers. The highest results appear to have been obtained by Givner et al using Bauld's method. Morse et al also used Bauld's procedure. The greatest contribution to Givner's figures was made by estriol. The range of values found in the present study agreed quite well with those of most of the remainder of the workers although there was a tendency for the ratio of estriol to estrone to be higher in our laboratory. Although the estradiol levels were consistently low some was normally detected, in agreement with the others except Preedy and Aitken. Total ring D α-ketols measured by method B ranged from 0.6 to 11.0 μg/24 hours with an average of 3.8 μg/24 hours for the fifteen control males of Table III.

Table IV contains data for urinary estrone, estradiol and estriol in post-menopausal females (in the present work, at least five years post-menopausal). The results obtained in this laboratory, using method B, agreed well with those of other workers although they were higher than those reported by Jull et al. Using method B the total ring D α-ketols ranged from 0.1 to 2.0 μg/24 hours with

TABLE III

ESTRONE(E_1), ESTRADIOL(E_2) AND ESTRIOL(E_3) MEASURED IN MALE URINE BY VARIOUS INVESTIGATORS

(μg./24 hours with range or standard deviation)

Authors	No. of subjects	Age (years)	E_1	E_2	E_3	Total
Brown[15]	29	20 - 50	5.4 (3.0-8.2)	1.5 (0.0-6.3)	3.5 (0.8-11.0)	10.4 (6.0-17.8)
Bersohn & Oelefse[16]	21	20 - 48	4.3 (1.7-9.8)	1.1 (0.0-3.1)	2.6 (0.6-9.9)	8.0 (3.8-15.4)
" "	20	45 - 65	6.3 (2.8-12.5)	2.1 (0.5-3.8)	6.0 (1.3-12.7)	14.4 (5.6-21.1)
Salokangas & Bulbrook[5]	4	---	2.3 (1.5-4.2)	1.0 (0.6-1.4)	3.1 (1.3-7.4)	6.4 (3.5-13.0)
Givner et al[10]	7	---	3.9 (3.2-4.8)	2.9 (1.0-4.8)	10.0 (6.0-20.0)	16.8 (10.3-28.4)
Morse et al[17]	7	Mean 26	7.2±2.3	2.6±0.7	5.5±2.4	15.2±4.0
"	16	Mean 71	3.2±1.7	1.5±1.0	5.6±3.8	10.4±5.1
Preedy & Aitken[18]	10	22 - 57	4.3 (1.4-7.9)	<0.5	2.1 (<0.5-5.4)	6.4 (2.2-13.3)
Present study	15	17 - 72	3.5 (0.8-7.1)	0.9 (0.4-1.7)	6.1 (1.5-10.4)	10.5 (3.8-17.1)

TABLE IV

ESTRONE(E_1), ESTRADIOL(E_2) AND ESTRIOL(E_3) MEASURED IN URINE OF POST-MENOPAUSAL FEMALES BY VARIOUS INVESTIGATORS

(μg./24 hours with range or standard deviation)

Authors	No. of subjects	Age (years)	E_1	E_2	E_3	Total
Brown[19]	22	5 yrs. + post-men.	1.4* (0.4-5.2)	0.3 (0.0-1.5)	4.1* (1.0-16.2)	6.0* (1.9-19.1)
McBride[20]	7	mean 59	1.8 (0.0-7.2)	0.6 (0.0-5.6)	3.3 (0.0-10.8)	5.7
Bulbrook[21]	16	----	1.8	0.9	2.4	5.1
Jull et al[22]	100	40 - 76	1.5±0.8	0.3±0.6	2.1±1.7	3.9
Present study	12	52 - 72	1.2 (0.2-3.1)	0.4 (0.0-0.9)	4.1 (0.6-10.4)	5.7 (1.6-13.3)

* Geometric means and ranges (P = 0.05).

an average of 1.2 μg/24 hours.

In Table V are shown levels of five estrogen fractions, measured by method B, in the urine of some males and post-menopausal females. It should be noted that these figures, besides those obtained by method B and shown in Tables III and IV, have not been corrected in any way for methodological losses.

DISCUSSION

The original method of Bauld[2] was claimed to possess useful sensitivity down to about 3 μg of either estrone, estradiol or estriol/24 hours. Below that level considerable errors might be anticipated. Even above this level, however, it would appear that overestimations may occur. Earlier results obtained by method A for estrone and estriol in this laboratory[11] were in the general range obtained by other workers using other procedures but the average figures appeared to be somewhat high. This may have been due to overestimation by method A although in the present work the most obvious error was in the estradiol fraction. Method B, while yielding totals similar to those found by other investigators, usually showed a higher estriol: estrone ratio for males than was found by others. Whether this is due to the presence of an additional compound in the estriol fraction, or to the selection of the subjects studied, some being hospitalized, is uncertain. Gallagher has claimed that estriol: estrone ratios are generally less than unity in healthy males and greater than unity in healthy

TABLE V

FIVE ESTROGEN FRACTIONS IN HUMAN URINE*

(μg./24 hours)

Subject (age)	E_1	E_2	16α-OHE_1	16-KE_2	E_3	Total
Male (19)	3.0	0.8	0.9	0.6	1.5	6.8
Male (27)	3.6	1.7	2.4	2.0	4.7	14.4
Male (32)	0.8	0.4	0.9	0.9	6.5	9.5
Male (42)	2.6	1.1	2.4	3.8	10.4	20.3
Male (49)	1.3	0.4	0.7	0.4	2.1	4.9
Male (50)	1.5	0.8	2.0	1.6	6.8	12.7
Male (72)	3.2	1.0	1.1	1.0	5.4	11.7
Female (50)	2.3	0.5	0.9	1.3	6.1	10.9
Female (63)	0.9	0.4	0.8	1.3	6.5	9.7
Female (64)	0.2	0.1	0.5	0.3	2.3	3.3
Female (70)	0.3	0.1	0.7	0.5	1.2	2.6

* E_1 = estrone, E_2 = estradiol, 16α-hydroxyestrone, measured as estriol reduction product of the ring Dα-ketols, 16-KE_2 = 16-ketoestradiol-17β, measured as 16-epiestriol reduction product of the ring Dα-ketols, E_3 = estriol.

females[23]. Method B gives an average ratio of 1.7 for the male subjects studied and 3.4 for the females. Thus, it might be said that such a trend is indicated.

The sodium borohydride reduction products of the ring D α-ketols indicate that approximately equal amounts of estriol (arising from 16 α-hydroxyestrone) and 16-epiestriol (arising from 16-ketoestradiol-17β and/or 16β-hydroxyestrone) are probably excreted. The total amount of ring D α-ketols is approximately the same as for estrone. This is in agreement with earlier work in this laboratory[11]. Accurate measurements of these compounds must, however, await improved methodology.

The following trivial names have been used in the text:

Estrone = 3-hydroxy - 1,3,5 (10) - estratrien - 17 - one.

Estradiol-17β = 3, 17β-dihydroxy - 1,3,5 (10) - estratriene.

Estriol = 3, 16α, 17β-trihydroxy - 1,3,5 (10) - estratriene.

16-Ketoestradiol-17β = 3, 17β-dihydroxy - 1,3,5 (10) - estratrien-16-one.

16α-Hydroxyestrone = 3, 16α-dihydroxy - 1,3,5 (10) - estratrien-17-one.

16β-Hydroxyestrone = 3, 16β-dihydroxy - 1,3,5 (10) - estratrien - 17-one.

16-epiEstriol = 3, 16β, 17β-trihydroxy - 1,3,5 (10) - estratriene.

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SECTION B

URINARY OESTROGEN LEVELS WITHOUT REGARD TO SPECIFIC MODES OF CONJUGATION

SOME ASPECTS OF THE ENZYMIC HYDROLYSIS OF URINARY 17-KETOSTEROID CONJUGATES[1]

R. HOBKIRK AND J. J. COHEN

Abstract

Four enzyme preparations containing β-glucuronidase, of bacterial, mammalian, and molluscan origin, have been shown to be equally effective in liberating 17-ketosteroids (17-KS) of the 5β-(etiocholane) configuration in normal urine. The bacterial preparation releases steroids of the 5α-(androstane) configuration more rapidly than do the molluscan enzymes and with much greater ease than does the liver enzyme. In view of the data obtained it seems unlikely that the striking difference between the bacterial and liver enzymes can be due to the hydrolysis of some labile conjugate, such as sulphate, by the former and not by the latter. Possibilities that the difference is due to the hydrolysis of an unknown type of urinary conjugate by the bacterial preparation, or to the low specificity of the bacterial β-glucuronidase, are discussed. The high degree of hydrolysis of 17-KS conjugates by the bacterial enzyme followed by solvolysis suggests this as a most useful hydrolytic procedure.

Introduction

It is now generally recognized that the measurement of individual neutral urinary 17-ketosteroids (17-KS) as a test of endocrine function is much superior to the group assay of total 17-KS (1–6). In order to separate and measure these steroids, which are present in urine as glucuronide and sulphate conjugates, a hydrolytic procedure that results in no artifact formation is required as a preliminary step (5, 7). This involves the use of enzyme preparations from various sources containing β-glucuronidase and in some cases sulphatase (8–12). Quantitative hydrolysis of urinary 17-KS sulphates by sulphatases is unlikely, because of the inhibitory effect of certain ions on these enzymes and to stereochemical considerations (13–15), but these labile conjugates are conveniently split by certain mild acid treatments (7, 16).

It has been reported that mammalian liver β-glucuronidase was unable to liberate the same amounts of 17-KS from urine as did hot acid hydrolysis (17), especially where steroids of the 5α-(androstane) configuration were concerned. An extract from the snail *Helix pomatia* did not show this difference to such an extent (18), and an enzyme preparation from the limpet *Patella vulgata*, together with mild acid hydrolysis, has been shown to liberate a very high percentage of urinary 17-KS (5, 19).

To our knowledge, a detailed investigation of the liberation of individual urinary 17-KS by a bacterial enzyme preparation has not been reported upon. A study has therefore been made comparing the ability of four enzyme preparations of bacterial, mammalian, and molluscan origin to hydrolyze urinary 17-KS conjugates. Special attention has been paid to the hydrolysis of conjugated 5α-17-KS by the bacterial and mammalian liver enzymes. The steroids measured were the three main C19 11-oxygenated metabolites of adrenocortical

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steroids, 3α,11β-dihydroxyetiocholan-17-one (11β-hydroxyetiocholanolone),* 3α,11β-dihydroxyandrostan-17-one (11β-hydroxyandrosterone) and 3α-hydroxyetiocholane-11,17-dione (11-ketoetiocholanolone), together with the 11-deoxy-steroids 3α-hydroxyetiocholan-17-one(etiocholanolone) and 3α-hydroxyandrostan-17-one (androsterone), which are metabolites of both adrenal and testicular origin. Beside these, urinary 3β-hydroxy-Δ5-androsten-17-one(dehydroepiandrosterone), which is probably of adrenal origin, was measured.

Materials and Methods

Urine Collection

Complete 24-hour specimens were collected from normal males and females. Collections were made in clean polyethylene bottles containing no preservative. Three urine pools were prepared, one from female urine, the others (male urines 1 and 2) from different male specimens. Three other 24-hour male urines were used individually (male urines 3, 4, and 5). All urines were kept in the deep-freeze until analyzed.

Chemicals

Organic solvents, with the exception of ethanol, were Reagent Grade. Suitable purification was carried out where necessary. Girard Reagent-T (Brickman Co.) was used as supplied. *m*-Dinitrobenzene was purified by an accepted procedure (20). Aluminum oxide (Woelm, almost neutral, grade I) was adjusted to contain 4.5% by weight of water. All other reagents were Analytical Reagent Grade and were used as supplied.

Enzyme Preparations

Bacterial β-glucuronidase — Sigma Chemical Co.

Mammalian liver β-glucuronidase (Ketodase) — Warner Chilcott Laboratories Inc.

Molluscan β-glucuronidase (Glusulase), also containing sulphatase, prepared from the snail *Helix pomatia* — Endo Laboratories Inc.

Molluscan β-glucuronidase, also containing sulphatase, prepared from the common limpet *Patella vulgata*; powder B of Dodgson and Spencer (10).

The unit of activity of these preparations was based on their β-glucuronidase contents and was defined as that amount of enzyme which liberated 1 μg of phenolphthalein from a 0.01 *M* solution of phenolphthalein glucuronide when incubated for 1 hour at 37° C. The pH of the medium was 6.5 for the bacterial preparation, 5.0 for the liver enzyme, 5.2 for the snail preparation, and 4.6 for the limpet material.

Hydrolysis and Extraction of the Steroids

Volumes of urine containing 2–3 mg of total 17-KS, as measured after hot acid hydrolysis, were incubated for 12-, 48-, and 96-hour periods at 37° C with each of the enzyme preparations. Conditions of pH and enzyme concentrations are given in Table I. For the bacterial and snail enzymes urine pH was adjusted

* For the sake of convenience the following abbreviations in nomenclature are used in this paper: 11β-hydroxyetiocholanolone, 11-OH-E; 11β-hydroxyandrosterone, 11-OH-A; 11-ketoetiocholanolone, 11-KE; etiocholanolone, E; androsterone, A; dehydroepiandrosterone, DHA.

with dilute NaOH or glacial acetic acid (18, 21). Acetate buffer was used for the other two preparations (3, 5). The enzyme concentrations in Table I were those found to achieve apparently maximum release of total urinary 17-KS after incubation for 48 hours with normal urine.

Following incubation, neutral ether extracts were prepared in readiness for Girard separation. Solvolytic cleavage of labile 17-KS conjugates, e.g. sulphates, was performed on the urines both before and after enzyme hydrolysis. In the latter case, urine, after extraction of 17-KS liberated by the enzymes, was made 2 *N* with 50% H_2SO_4, the subsequent steps being those originally described (16).

Where hot acid hydrolysis was performed following solvolysis the acidified urine from the latter step, together with the alkaline washes of the ether extract from the enzyme-hydrolyzed urine, was refluxed for 1 hour on a steam bath.

All analyses were performed in duplicate.

Separation and Measurement of 17-KS

The dried extracts were separated into ketonic and non-ketonic fractions by a modification of the Girard reaction (22) performed at room temperature. The ketonic fractions were then separated into their six main 17-KS by alumina column and paper chromatography, essentially as described by Brooks (19). Steroid containing zones on the paper chromatograms were eluted and analyzed by a modified Zimmermann reaction (23), reading optical densities at 480, 520, and 560 mμ on a Beckman Model B or Unicam SP600 spectrophotometer. Allen's equation (24) was used to correct for non-specific chromogens. Each steroid was initially measured in terms of DHA and then corrected for the appropriate extinction coefficient. Portions of the ethanolic eluates from paper chromatograms were treated with concentrated H_2SO_4 and scanned between 220 and 600 mμ (25) on the Beckman Model DK-2 recording spectrophotometer. The spectra were compared with those obtained using pure steroids.

Reliability of the Method

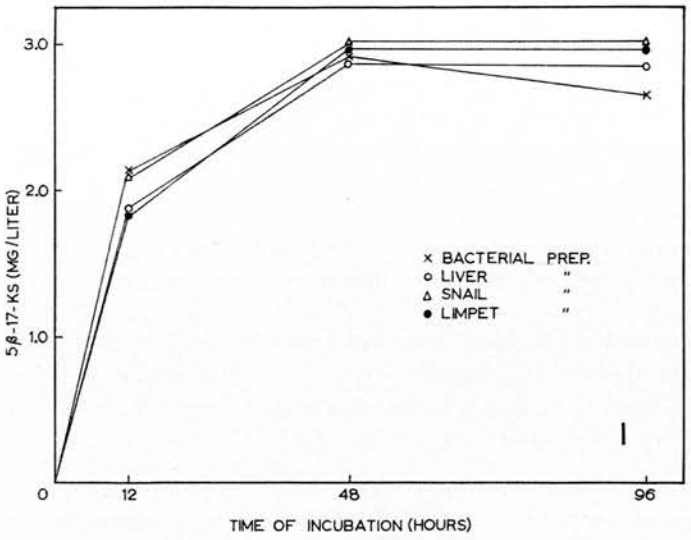
Recovery of pure steroids added to hydrolyzed urine and taken through the whole procedure was 87% $\pm$11 (standard deviation).

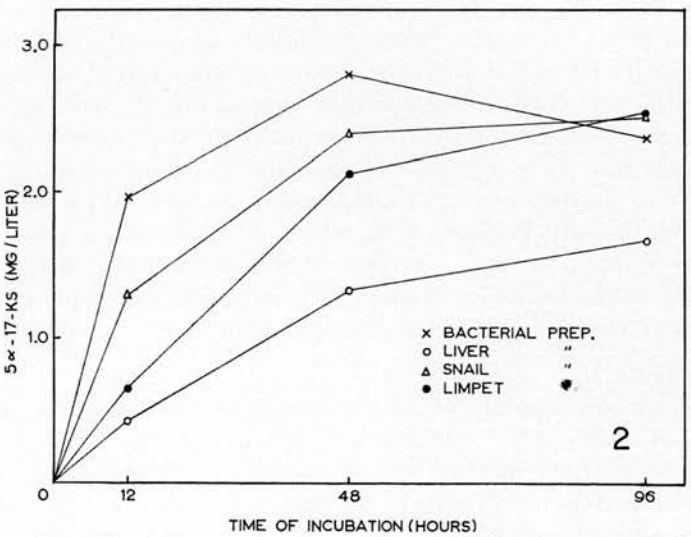
The precision of the method was assessed by calculation of an estimate of the standard deviation (s) from the difference of duplicate determinations in a series of measurements s being $\sqrt{\{S\ (d^2)/2N\}}$ where d is the difference between duplicates and N is the number of duplicate analyses performed (26). For the range 0.10 – 0.99 mg of any 17-KS/liter of urine, $s = 0.076$, while for the range 1.00–4.00 mg/liter, $s = 0.20$. The lower limit of sensitivity of the method is 0.15 mg of any one 17-KS/liter of urine.

Results

Release of 5α- and 5β-17-KS by Enzymes

Figure 1 shows total 5β-17-KS (E + 11-OH-E + 11-KE) liberated in normal female urine during incubation for 12, 48, and 96 hours with each of the four enzyme preparations. Good agreement for the different enzymes existed at each time and the same was true for the individual 5β-17-KS (not shown).





FIGS. 1 and 2. Effect of incubation time on the liberation of total 5β-17-KS (E+11-OH-E+11-KE) (Fig. 1) and 5α-17-KS (A+11-OH-A) (Fig. 2) in normal human female urine by β-glucuronidase-containing preparations of bacterial, mammalian liver, and molluscan origin.

However, the bacterial preparation caused much greater hydrolysis of conjugated 5α-17-KS (A + 11-OH-A) in the same urine than did the liver enzyme (Fig. 2) under the experimental conditions employed. The bacterial preparation also achieved a more rapid release of these steroids than did the snail and limpet enzymes (Fig. 2, Table I). A tendency for 17-KS levels to decrease when incubated for 96 hours with bacterial enzyme may have been due to bacterial destruction since no antibiotics were used. In each of three separate urine pools about two and five times more A and 11-OH-A, respectively, were released by

TABLE I

Release of urinary androsterone by enzymes, results expressed as mg/liter of urine

Enzyme	Female urine			Male urine 1		
	Time of incubation (hours)					
	12	48	96	12	48	96
Bacterial, pH 6.5, 75 units/ml	1.70	2.25	1.80	2.34	2.53	1.40
Liver, pH 5.0, 300 units/ml	0.35	1.20	1.45	0.25	1.04	1.40
Snail, pH 5.2, 500 units/ml	1.20	2.20	2.20	1.07	1.95	2.67
Limpet, pH 4.6, 750 units/ml	0.50	1.85	2.20	0.74	1.57	1.99

75 units/ml of bacterial preparation than by 300 units/ml of liver enzyme (Table III). An increase in liver enzyme concentration to 750 units/ml still failed to equal the performance of the bacterial enzyme. E and 11-OH-E levels were not significantly different in these urines regardless of whether bacterial or liver enzymes were employed.

Hydrolysis of Labile 17-KS Conjugates

Minimal amounts of DHA (if any) were freed by bacterial and liver enzymes (Table II) while the snail and limpet preparations hydrolyzed slightly more. This might be expected because of the sulphatase content of the latter two.

TABLE II

Liberation of DHA in normal urine;* results expressed as mg/liter of urine

Enzyme†	Free DHA after incubation with enzymes		DHA liberated by solvolysis following incubation with enzymes		Total DHA (sum of previous two columns)	
	Male urine 1	Female urine	Male urine 1	Female urine	Male urine 1	Female urine
Bacterial	0.21	0	1.33	0.32	1.54	0.32
Liver	0.24	0	1.55	0.32	1.79	0.32
Snail	0.38	0.12	1.22	0.18	1.60	0.30
Limpet	0.50	0.29	0.86	(?)	1.36	0.29

*Solvolysis of the original urine before enzyme action showed male urine 1 and the female urine to contain 1.80 and 0.34 mg DHA/liter, respectively.
†Conditions of incubation as in Table I; time of incubation, 48 hours.

Virtually all the urinary DHA (as measured prior to enzyme action) was liberated by solvolysis of the urine after incubation with bacterial or liver preparation. Of the other 17-KS measured only E and A were detected in the free form after solvolysis of the original urine from both males and females. Out of 0.21 mg of A measured in this way in a female urine pool, 0.18 and 0.22 mg remained to be freed by solvolysis following 48 hours' incubation with bacterial and liver enzyme respectively. Corresponding results for a male urine pool were 1.03 mg of A before enzyme action with 0.72 and 0.92 mg released by solvolysis after bacterial and liver enzyme action. These results are interpreted as meaning that the activities (if any) of the bacterial and liver enzymes towards labile 17-KS conjugates are similar.

TABLE III

Liberation of urinary 17-KS by bacterial and mammalian liver enzymes in 48 hours; results are expressed as mg/liter of urine*

	Female urine			Male urine 1			Male urine 2			
Enzyme†	E	A	11-OH-A	E	A	11-OH-A	E	11-OH-E	A	11-OH-A
Bacterial	2.45 (2.05–2.85)	2.25 (1.85–2.65)	0.56 (.41–.71)	2.25 (1.85–2.65)	2.53 (2.13–2.93)	1.28 (.88–1.68)	3.20 (2.80–3.60)	0.40 (.35–.55)	4.23 (3.83–4.63)	1.29 (.89–1.69)
Liver	2.50 (2.10–2.90)	1.20 (.80–1.60)	0.10 (0–.25)	2.64 (2.24–3.04)	1.04 (.64–1.44)	0.26 (.11–.41)	2.88 (2.48–3.28)	0.30 (.15–.45)	2.40 (2.00–2.80)	0.29 (.14–.44)
Liver (750 units/ml)		—			—		3.34 (2.94–3.74	0.43 (.28–.58)	3.27 (2.87–3.67)	0.63 (.48–.78)

*Fiducial range ($p = 0.01$) is shown in parentheses for each result.
†Conditions of incubation as in Table I except where indicated.

Hydrolysis of Total 17-KS Conjugates

Complete liberation of 17-KS in normal human urine was achieved by incubation for 48 hours with bacterial enzyme followed by solvolysis. Table IV gives the levels of six 17-KS fractions freed by this procedure. Hot acid hydrolysis after solvolysis resulted in a small positive measure for total 17-KS but in no case was a typical Zimmermann-positive zone seen on paper chromatograms of these extracts.

TABLE IV

Amounts of individual 17-KS released in normal male urine by bacterial enzyme followed by solvolysis;* results expressed as mg/24 hours

Urine specimen	Age (years)	DHA	E	A	11-OH-E	11-OH-A	11-KE
Male urine 3	22	0.59	2.96	4.79	0.31	0.88	0.45
Male urine 4	22	2.10	2.68	3.47	0.58	1.29	1.00
Male urine 5	55	0	0.89	0.72	0.20	0.65	0.46

*Individual 17-KS were not released in measurable amount by further hot acid hydrolysis.

Discussion

Incomplete hydrolysis of 5α-17-KS conjugates by mammalian liver β-glucuronidase has been reported by Wotiz *et al.* (17). In order to explain their findings these workers suggested the presence of non-glucuronide conjugates (possibly sulphates) in the urine. However, separation of 17-KS glucuronide and sulphate fractions from urine has shown little or no 11-OH-A to be present as the latter type of conjugate (5, 19, 27), which is in agreement with the results in the present work. Also, any likelihood that the difference in activity between the bacterial and liver enzymes might be due to sulphatase activity in the former is further diminished by the finding that neither of these preparations liberated much of the DHA or A present as labile conjugate. Phosphatase activity has been shown to occur in the snail enzyme preparation (28), and a complex containing DHA and phosphate has been identified in plasma (29). If steroid phosphates were present in the urine, however, they would presumably constitute part of the labile conjugate fraction. Therefore any possibility that phosphatase might play an important part in the bacterial enzyme activity could be dismissed on the same grounds as for the sulphatase. An alternative explanation would be to postulate the occurrence of a hitherto unrecognized 5α-17-KS conjugate, of a non-labile nature, capable of being hydrolyzed by a similarly hypothetical enzyme constituent of the bacterial preparation. Experiments to test this possibility are at present being conducted in this laboratory. The existence of a urinary conjugate of 11-OH-A, resistant to hydrolysis by enzyme or mild acid treatment, has been reported (7). Although inhibitors of β-glucuronidase (30, 31) in urine might be considered to account for the lower activity of the liver preparation, the preferential inhibition of 5α-17-KS conjugate hydrolysis would still remain to be explained.

Besides these possible explanations it is conceivable that the bacterial β-glucuronidase is generally a less specific enzyme than the mammalian liver one and therefore able to achieve more rapid hydrolysis of a greater variety of

glucuronides. That the action of the bacterial enzyme on 17-KS conjugates is basically a manifestation of glucuronidase activity is supported by the essentially complete hydrolysis of urinary conjugates, primarily of the 5α-17-KS type, by a sufficiently long incubation with the purified liver β-glucuronidase (31, 33). The complete hydrolysis achieved by the bacterial enzyme plus the solvolytic procedure, together with the good agreement in individual 17-KS levels obtained when compared with the results of other workers (3, 5, 19) suggests this as a useful hydrolytic method.

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URINARY ESTROGEN EXCRETION IN NORMAL AND DIABETIC PREGNANCY*†

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ABSTRACT

Urinary levels of estriol, estrone and estradiol-17β (estradiol) increase during the last 20 weeks of pregnancy in a qualitatively similar fashion in both the normal and diabetic states. The relative amounts of these estrogens found in the urine suggest that they arise from the same source in both groups according to the transformation, estradiol$\rightleftharpoons$estrone$\rightarrow$estriol. It also appears likely that there is an additional source of estriol in the two groups, particularly in late pregnancy. There is a tendency for estriol levels to be lower in diabetic than in non-diabetic pregnancy. Considerable variability in the relative amounts of these 3 estrogens from one pregnancy to another, both diabetic and normal, suggests the occurrence of a varying pattern of estrogen metabolism from one individual to another.

INTRODUCTION

COMPLICATIONS during pregnancy in patients with diabetes mellitus, particularly the high incidence of intra-uterine death, have been recognized for some time. Despite this, little precise information is available regarding the biochemical aspects of the problem. Abnormal levels of gonadotropins and pregnanediol have been reported in diabetic pregnancy (1) but the field of estrogen metabolism has been largely neglected. Rubin *et al.* (2), using a chemical method of assay, reported data on

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a few patients showing that urinary estrogen excretion may be normal in diabetic pregnancy uncomplicated by pre-eclamptic toxemia, but low when toxemia is present.

The present investigation was undertaken with a view to ascertaining whether or not diabetic pregnancy has associated with it any abnormality in the urinary estrogen pattern. To this end estriol, estrone and estradiol-17β (estradiol) were measured by an established chemical procedure (3). The biochemical aspects of the study are presented in this paper.

MATERIAL AND METHODS

Subjects

The control group consisted of 10 pregnant women exhibiting no clinical abnormalities. The diabetic group, none of whom exhibited pre-eclamptic toxemia, numbered 14 pregnant women with varying degrees of diabetes mellitus, all of whom were maintained with insulin. None of the subjects, normal or otherwise, was receiving any other type of medication. The two groups were studied between the twentieth week of pregnancy and delivery. Estrogen determinations were made weekly in the normal group, and from 3 to 15 estimations were carried out on each of the diabetic patients over the twenty-week period.

Urine collection

Urine was collected as 24-hour specimens in specially cleaned polyethylene bottles without preservative. Estrogen determinations were made as soon as possible thereafter. When delay was necessary, the urine was stored in the refrigerator at 4°C. until the assay could be performed.

Estrogen determination

Estriol, estrone and estradiol were measured chemically by the method described by Bauld (3), or a modification of this procedure (4). An aliquot of from 10 to 50 ml. of the 24-hour urine specimen was taken for analysis, depending upon the stage of pregnancy. Diabetic urines were diluted 1:10 with distilled water prior to acid hydrolysis in order to overcome the destructive effect of glucose on the urinary estrogens (4). When the larger urine aliquots were necessary, the original Bauld procedure was followed after hydrolysis. This involves partition of a phenolic extract between benzene and water, with subsequent partition chromatography of the benzene (estrone-estradiol) fraction and saponification of the aqueous (estriol) extract. The next stage consists of saponification of the separated estrone and estradiol, and partition chromatography of the estriol fraction. The 3 separated estrogens are then measured spectrophotometrically by the Kober reaction. All analyses were performed on duplicate urine aliquots. When small volumes of urine could be used (*e.g.*, 10 ml.), saponification of the estrone and estradiol fractions and chromatography of the estriol fraction were omitted (4, 5). The reliability of these chemical procedures as carried out in this laboratory has already been established for urines not containing glucose, as discussed elsewhere (5, 6).

To obtain information on recovery of the 3 estrogens in the presence of glucose, 10-ml. portions of 7 nonpregnancy human urines were subjected to acid hydrolysis after 1:10 and 1:25 dilution both with and without the addition of 2–4 per cent glucose to the original urine. Prior to hydrolysis, 25–50 μg. of estriol, 10.9 μg. of estrone and 10 μg. of es-

tradiol were added to each diluted aliquot. A total of 32 duplicate analyses was performed. Percentage recoveries of estriol (with standard deviations) at 1:10 dilution, with and without 2 per cent glucose, were 76 ± 3.4 and 79 ± 3.9, respectively. Corresponding values for the recovery of estrone were 87 ± 1.9 and 92 ± 2.1, and for estradiol 77 ± 3.1 and 83 ± 2.5. At 1:25 dilution there was no significant increase in recovery. Thus for estriol, with and without 2 per cent glucose, the percentages were 78 ± 1.0 and 79 ± 3.7. In 2 experiments in which 4 per cent glucose was added prior to 1:10 dilution, the mean recovery of added estriol was 75 per cent. In the insulin-controlled diabetic group, the highest level of urinary sugar recorded was 2 per cent.

Recovery of estriol and estradiol was somewhat lower than might have been expected from earlier work (5). The foregoing results, however, make it legitimate to compare levels of the 3 estrogens in diabetic and in nondiabetic pregnancy urines diluted 1:10 before acid hydrolysis. Moreover, since further dilution does not significantly influence recovery of added estrogen, it is likely that optimum levels of estriol, estrone and estradiol are measured at 1:10 dilution. It is accepted that the effect of acid hydrolysis on free estrogens in the presence of glucose is not necessarily the same as on urinary estrogen conjugates. Until these conjugates have been characterized, however, more precise information will not be available.

RESULTS

Normal pregnancy

The urinary levels of estriol, estrone and estradiol between the twentieth and fortieth weeks of pregnancy are shown in Figures 1, 2 and 3. Estrone and estradiol showed parallel rises, each increasing by about 165 per cent during the period studied. Estriol showed an even greater average increase of about 320 per cent during this time. These findings, both qualitatively and quantitatively, are in good general agreement with those reported by Brown (7). The average ratio of estrone to estradiol (referred to hereafter as E_1/E_2) calculated at 20, 25, 30, 35 and 40 weeks varied only slightly, lying in the range 3.2–4.7. Incidentally, the E_1/E_2 ratios for individual pregnancies (not shown) were in the relatively narrow range of 2.0–6.0. The average ratio of estriol to estrone plus estradiol (referred to hereafter as E_3/E_1+E_2) was in the range 7.0–9.0 at 20 and 25 weeks and then increased sharply to 13.0 at 30 weeks. This emphasizes the more rapidly increasing excretion of estriol at that particular time.

Detailed consideration of estrogen levels in the individual normal pregnancies is not merited here, but it is of interest to note the distinctly different E_3/E_1+E_2 ratios for different pregnant subjects. An example is shown in Table 1. One of these pregnant women (Subject A) had a low ratio of 3.0–5.0 during the greater part of the 20-week period, with a rise in the later stages, whereas the other (Subject B) had a much higher ratio throughout, with a similar tendency toward an eventual increase. In all of the 10 normal cases studied, there was a similar late and upward trend in this ratio, despite its variable magnitude.

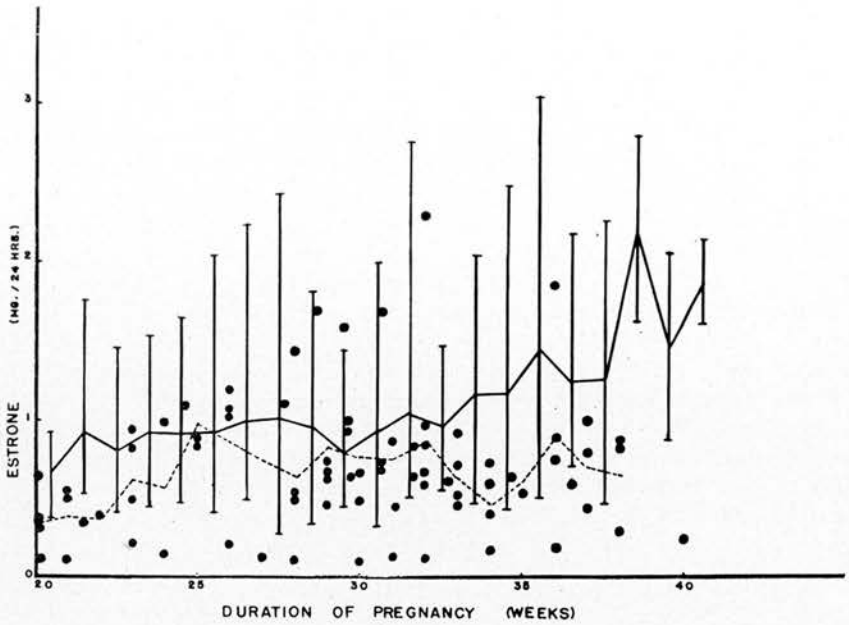

FIG. 1. Excretion of estrone during pregnancy in normal (———) and in diabetic (– – – –) subjects. Vertical lines represent the range of estrone measurements in 10 normal pregnant women, the mean weekly values being joined by an unbroken line. Each black dot represents an estrone measurement in any one of 14 diabetic subjects at various stages of pregnancy, the mean weekly values being joined by a broken line.

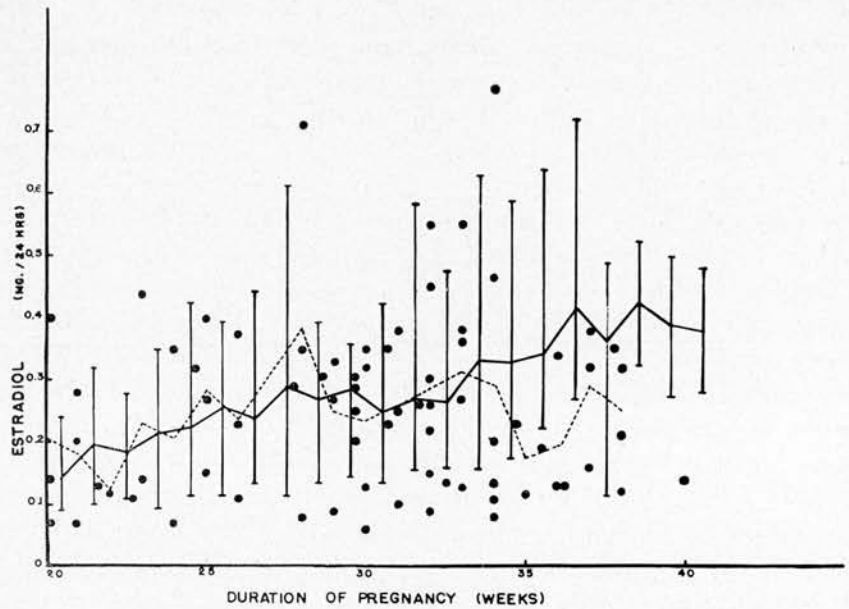

FIG. 2. Excretion of estradiol during pregnancy in normal (———) and in diabetic (– – – –) subjects. For key to other symbols (as applied to estradiol), see legend to Figure 1.

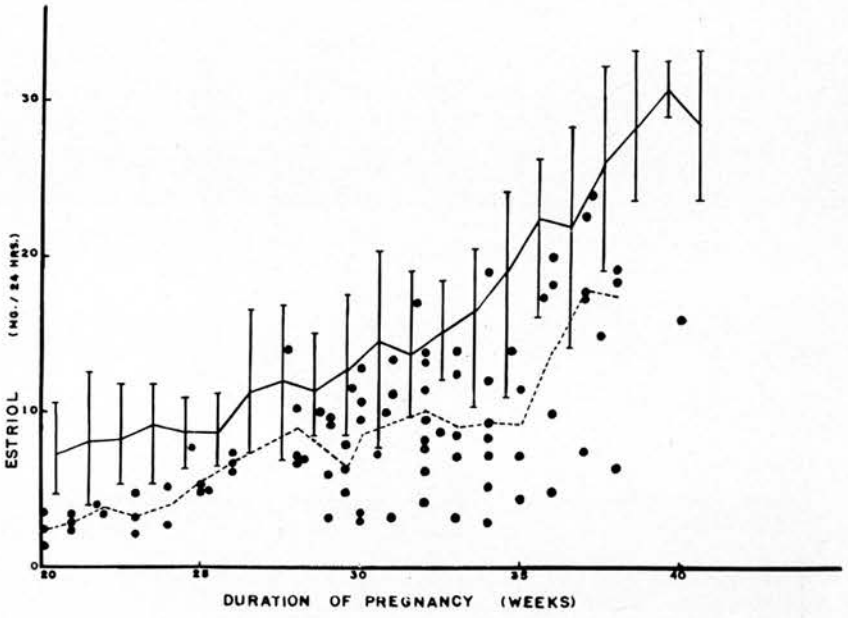

FIG. 3. Excretion of estriol during pregnancy in normal (———) and in diabetic (– – – –) subjects. For key to other symbols (as applied to estriol), see legend to Figure 1.

Diabetic pregnancy

In Figures 1, 2 and 3, the results for the 3 urinary estrogens in the diabetic group are shown along with results obtained in the normal group. This type of diagram was designed to show the range of urinary estrogen levels in the diabetic subjects, and not the trend of excretion in individuals.

Estrone and estradiol values in the diabetic group showed considerable scatter, but were mostly within the normal range, which was also wide. Individual determinations below the normal range in Figures 1 and 2 represent estrone and estradiol levels in 2 of the diabetic pregnancies studied. Estrone levels at 28 to 35 weeks in another diabetic patient (Subject C, Table 2; not included in Figure 1) were abnormally high. Omitting these high results, the average E_1/E_2 ratio for the diabetic group ranged from 3.2 to 3.5 when calculated at 20, 25, 30 and 35 weeks. This is similar to the ratio for the normal group. On the other hand, values for urinary estriol were almost wholly below the normal mean level, although about half of the results were within the normal range. The average E_3/E_1+E_2 ratio was lower in the diabetic group than in the normal women but the difference was not statistically significant. For the diabetic group, the ratio was in the range 5.3–7.6 between 25 and 30 weeks. As in the normal women, this ratio increased, and became 15.0 at 35 weeks. Thus there was a qualitative similarity between the two groups in this respect.

TABLE 1. URINARY E_3/E_1+E_2 RATIOS DURING 2 NORMAL PREGNANCIES

Subject	Week of pregnancy																	
	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
A	4.1	4.4	4.4	3.8	3.1	3.8	3.9	3.3	4.9	—	4.9	5.0	6.4	5.3	7.9	5.0	10.0	11.6
B	19.1	16.0	21.3	20.0	14.8	16.1	14.5	15.7	16.5	18.6	20.0	19.4	19.0	12.9	21.1	23.8	26.6	—

TABLE 2. URINARY ESTROGEN EXCRETION AND RATIOS DURING 3 DIABETIC PREGNANCIES

Subject	Estrogen excretion (mg./24 hrs.) and ratios	Week of pregnancy														
		26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
C	Estriol			10.3	9.7	10.8	11.2	11.5	14.0	19.0						
	Estrone			5.2	4.5	5.1	4.6	6.0	4.8	6.0						
	Estradiol			0.35	0.33	0.32	0.38	0.55	0.55	0.77						
	E_3/E_1+E_2			1.9	1.2	2.0	2.2	1.8	2.6	2.8						
	E_1/E_2			14.9	13.6	16.0	12.1	10.9	8.7	7.8						
D	Estriol				3.2	3.1	3.2	4.2	3.2	2.9	4.5	4.9	7.7	6.5		
	Estrone				0.68	0.69	0.87	0.99	0.92	0.74	0.54	0.76	1.0	0.83		
	Estradiol				0.09	0.13	0.10	0.15	0.13	0.11	0.12	0.13	0.16	0.12		
	E_3/E_1+E_2				4.2	3.8	3.3	3.7	3.1	3.4	6.8	5.5	6.6	6.8		
	E_1/E_2				7.6	5.3	8.7	6.7	7.1	6.7	4.5	5.8	6.3	6.9		
E	Estriol	6.8		7.0		3.5		5.8		7.2		10.0		19.4		16.0
	Estrone	0.21		0.13		0.10		0.13		0.19		0.19		0.30		0.14
	Estradiol	0.11		0.08		0.06		0.09		0.08		0.13		0.21		0.14
	E_3/E_1+E_2	21.2		33.4		21.8		26.4		26.6		31.2		38.0		57.1
	E_1/E_2	1.9		1.6		1.7		1.4		2.4		1.5		1.4		1.0

Individual diabetic pregnancies

A few comments on the estrogen excretion pattern in individual diabetic patients are pertinent. In Subject C (Table 2) the urinary estrone level was extremely high despite the normal estriol and estradiol levels. This resulted in a greatly elevated E_1/E_2 ratio of 7.8–16.0 over the period 28–34 weeks. Although Subject D (Table 2) also exhibited an elevated E_1/E_2 ratio, this was due to a lowered excretion of estradiol rather than to a raised excretion of estrone; in addition, the excretion of estriol was low. Subject E showed a low excretion of estriol over the period 30–34 weeks, with a fairly late increase to near-normal levels. This was associated with low excretion of estrone and estradiol throughout the study period, to the extent that the E_3/E_1+E_2 ratio was always above 21.2 and rose to 57.0 at 40 weeks.

DISCUSSION

The average urinary E_1/E_2 ratio over the period 20–40 weeks of pregnancy is remarkably constant (3.2–4.7), and is also approximately similar in both diabetic and nondiabetic pregnancies. This suggests that these 2 estrogens arise from a common source in both pregnancy groups—probably placental estrone or estradiol or both—since a number of investigators have reported that administration of estrone or estradiol to nonpregnant human subjects results in similar urinary E_1/E_2 ratios (6, 8, 9). Furthermore, it is likely that at least part of the urinary estriol has the same origin, since the metabolic relationship between the 3 "classic" estrogens (estradiol⇌estrone→estriol) is well known. However, as has been pointed out by others (9, 10), during pregnancy a great deal more estriol is excreted relative to estrone and estradiol than can be accounted for by metabolic transformation of the latter 2 estrogens, unless these are metabolized to a different extent in pregnancy as compared with the nonpregnant state. In this connection it has been reported that the conversion of exogenous estradiol to urinary estriol is greater than normal in prostatic cancer (11), myocardial infarction (8) and mammary cancer (12). It has also been claimed that in pregnancy the high urinary estriol excretion can be attributed to a greater transformation of estradiol (13), but this finding could not be reproduced by Pearlman *et al.* (14) after administration of deuterium-labeled estrone acetate in pregnancy. If there is no difference in this respect between the pregnant and nonpregnant states it would seem that there must be another source of urinary estriol, independent of estrone and estradiol metabolism, in both normal and diabetic pregnancy. Some support for this concept has arisen from *in vitro* observations on placental and other tissues showing that estriol may be formed from androgens (15, 16) and perhaps by a route which does not involve estrone or

estradiol (17). Such a mechanism might explain the finding in Subject E (Table 2) of a markedly increasing estriol excretion with continuing low estrone and estradiol excretions.

A variable E_3/E_1+E_2 ratio is not by any means confined to diabetic pregnancies. Throughout 2 pregnancies uncomplicated by diabetes (Subjects A and B, Table 1), the values were strikingly different. This suggests a tendency for the pattern of estrogen metabolism to vary from one pregnant woman to another. Also, high E_1/E_2 ratios, whether due to a high excretion of estrone (Subject C, Table 2) or to a low excretion of estradiol (Subject D, Table 2), suggest the possibility of a change in the extent of interconversion between estrone and estradiol under certain circumstances. These observations indicate the need for renewed and extensive studies of estrogen biosynthetic pathways in pregnancy, and application of the findings to disorders of the pregnant state.

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Urinary Estrogens in Pregnant Diabetics

A Clinical Correlation

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It is well known that pregnancy in the diabetic is subject to certain hazards, principally large fetal size, hydramnios and intra-uterine death. In an attempt to explain these abnormalities, measurements of hormones known to vary in pregnancy have been made and are recorded in the literature. Little attention has been paid to the measurement of estrogens. As early as 1937 Smith and Smith[1] reported the bioassay of serum and urinary estrogens in pregnant diabetics. The levels of estrogens were normal in some but were low in others. In this latter group elevated levels of gonadotrophins were often found and this combination was considered to indicate a poor prognosis. This work was extended by the original workers and White[2] and led to the claim that this unfavorable hormonal imbalance could be corrected by the administration of estrogens.

In 1946 Rubin et al.[3] reported chemical measurements of estrone, estradiol and estriol in the urine of five pregnant diabetics. The levels of estriol were normal in three and low in the two who showed evidence of toxemia. Measurement of estrone and estradiol were normal in all five. In contrast to the great clinical interest in the therapeutic use of estrogens there have been few estrogen measurements reported in pregnancy in the diabetic. This is probably due to the unreliability of bioassay and the technical difficulties of chemical analysis which existed. In recent years two different chemical methods were developed in Marrian's laboratory in Edinburgh for the estimation of the three classical estrogens: estriol, estrone and estradiol. Brown's[4] method was published in 1955 and Bauld's[5] in 1956. Both gave similar results and were much more reliable than earlier methods. This development gave a great impetus to the study of estrogen excretion in normal and abnormal pregnancy. Unfortunately it was soon observed that the presence of glucose in the urine interfered with the determinations. Hobkirk[6] found a way of circumventing the interference due to glucose and using his modification of Bauld's method published the findings in fourteen diabetic and ten normal pregnancies.[7] The purpose of this report is to extend the series and to describe the clinical features of the cases.

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MATERIAL

Measurements of estrone, estradiol and estriol were performed during the last half of twenty-six pregnancies in twenty-two diabetic patients. The average age of the patients was twenty-seven and a half years; the range was eighteen to thirty-nine years. The duration of diabetes varied from zero to twenty-eight years with a mean duration of ten and a half years. In two patients the diagnosis of diabetes was made during pregnancy: One required insulin and one dietary restriction only.

The outcome of pregnancy was as follows: There were three intra-uterine deaths, one associated with diabetic coma at twenty-seven weeks, and two in patients with renal damage; one death occurred during delivery of a breech; there was one unexplained death twelve hours after cesarean section and one death four days after delivery due to multiple congenital anomalies. Twenty of twenty-six are living.

The classification of the maternal state according to White's scheme is as follows: one class "A," five class "B," thirteen class "C," four class "D," and three class "F."

One patient received a variety of hormones for a short time in an unsuccessful attempt to control hydramnios and the estrogen determinations which may reflect this therapy have been omitted. One patient was given progesterone for intermittent bleeding.

The results of the estrogen analyses were not used to influence the clinical management of the cases.

RESULTS

Figure 1 shows graphically 156 estriol assays carried out on twenty-four-hour collections of urine in twenty-six diabetic pregnancies during the latter half of gestation. The mean is plotted as a solid line. The broken line represents the mean of fifteen normal pregnancies, the shaded area the range of the normal values. Approximately one half of the diabetic urines are below the normal range. There are a few values which are much higher. The mean is lower than normal but tends to rise almost to the normal level around the thirty-fifth week. In serial determinations on individual patients this seems to be the pattern most frequently seen.

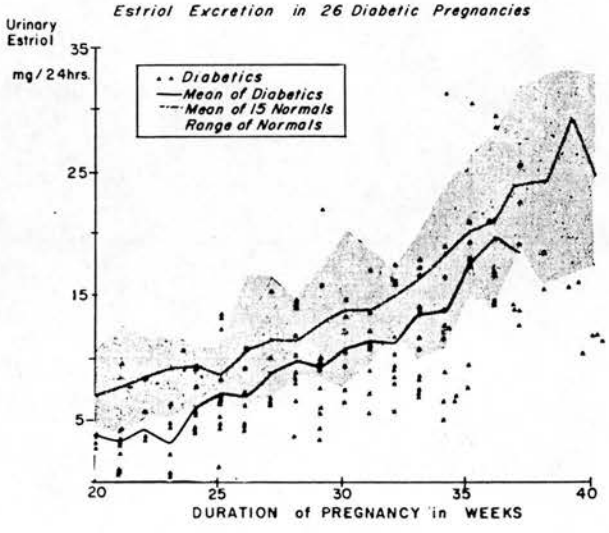

FIGURE 1

Figure 2 shows the values for urinary estradiol excretion in a series of seventeen diabetics and ten controls. Again the mean is plotted as a solid line. The normal mean is the broken line and the shaded area represents the range of normals. There is a considerable scatter of normals in view of the quantities being measured. The two diabetic values which are clearly above the normal range belong to the only patient in the group who developed pre-eclamptic toxemia.

Figure 3 shows the values for urinary estrone excretion in seventeen diabetics and ten controls. The same method of expressing results is used as in the previous figure. The three very high grouped values all belong to one patient, and this patient alone accounts for the elevation of the mean above the normal mean. No particular clinical abnormality was observed during the course of this patient's pregnancy.

There were two intra-uterine deaths in mothers with

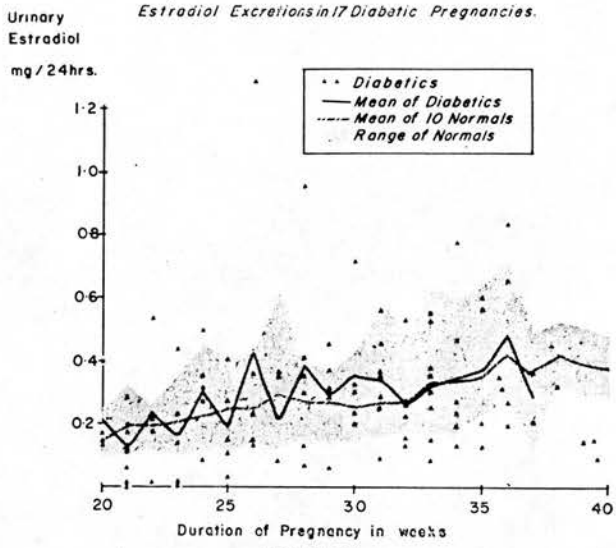

FIGURE 2

diabetic nephropathy. The pattern of estriol excretion in each case will be given in detail.

Case I.—F.S. This young woman of twenty-eight had been a diabetic for eleven years. This was her first pregnancy. She stated that she had been well until one year previously when she developed a large mass in the abdomen which proved to be her bladder. Investigation revealed that she had a neurogenic bladder most probably due to multiple sclerosis. When first seen at our hospital she was twenty-eight weeks' pregnant and had been catheterizing herself twice daily for the previous six months. The urine contained considerable pus and albumen; the blood urea nitrogen was and remained within normal limits during pregnancy. The pattern of estriol excretion is illustrated in figure 4. The first two values were obtained when she was first seen. She was sent back to her home in the country, and on her return three weeks later the first

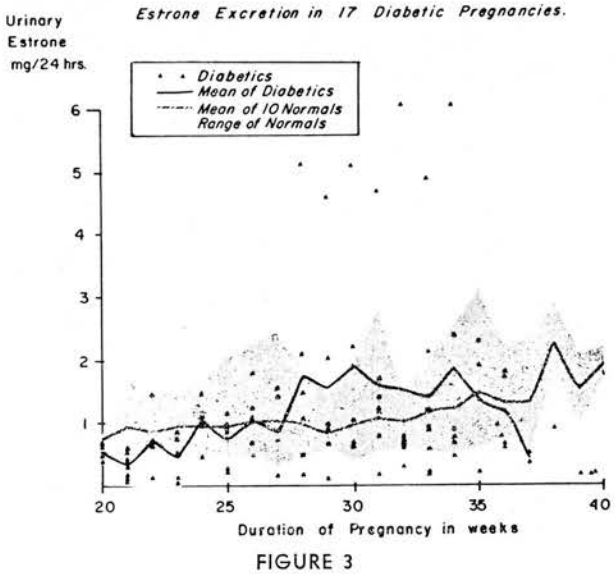

FIGURE 3

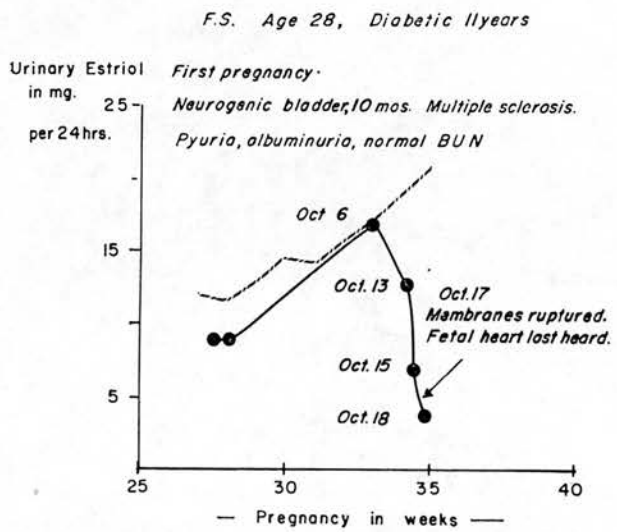

FIG. 4. Case I, F.S., estriol excretion in a diabetic pregnancy with intra-uterine death of the fetus.

analysis for urinary estriol on October 6 was normal—16.8 mg. per twenty-four hours. The obstetrician in charge estimated the size of the baby to be too small to justify section and it was decided to wait a bit longer. On October 10 she began to complain of nausea and ate less. Twenty-four-hour urinary estriol on October 13 was 12.6 mg., and on October 15 it was 6.9 mg. On October 17 the membranes ruptured spontaneously and the fetal heart sounds were last heard later the same day. A twenty-four-hour urine collection the following day, October 18, contained only 3.5 mg. estriol; the fetus was definitely dead. Although these events and determinations are reconstructed and reported chronologically, in fact the results of these assays only became available some days later.

Case II—I.D. This twenty-eight-year-old woman had been diabetic for thirteen years and showed evidence of albuminuria

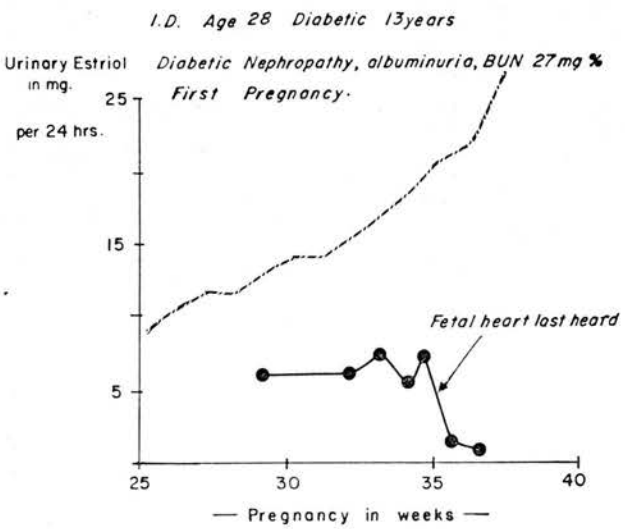

FIG. 5. Case II, I.D., urinary estriol excretion in a diabetic pregnancy resulting in fetal intra-uterine death.

and impaired renal function before her pregnancy. The urinary estriol excretion is represented in figure 5 as large dots. Although estriol excretion was about half of normal on the first estimation, this was not considered alarming; however, the failure of the estriol excretion to rise after thirty-three weeks was alarming. Because of the small fetal size, the risk of neonatal death was considered too great to warrant section. The fetal heart was last heard at thirty-five weeks. On subsequent visits, although the fetal heart sounds could not be heard, the patient insisted that she could feel movements. The analyses suggest that the baby died soon after the thirty-fifth week as the last two estriol values are incompatible with fetal survival at that stage of pregnancy.

DISCUSSION

Although knowledge of estrogen metabolism in normal pregnancy is still rudimentary, it seems reasonable to establish whether or not any striking differences exist in diabetics. It is known that when death of the fetus occurs estrogens are the first hormones to reflect this change.[8] It is thought that estradiol and/or estrone are produced by the placenta. Estriol is the principal estrogen in terms of quantity found in the urine. In the normal menstrual cycle and during the first trimester of pregnancy all three estrogens rise or fall together, and it is thought that estradiol and estrone are the precursors of estriol. However, in the last trimester urinary estriol increases so rapidly and to such a high level relative to the other two that it has been suggested that there must be another precursor. Recently Brown and Preedy[9] have made the alternative suggestion that the renal clearance of estriol increases towards the end of pregnancy relative to estrone as a result of increased tubular secretion. In very recent years a considerable number of metabolites of estrogens in the human subject have been reported. These include 2 methoxyestrone 16 epiestriol and the ring D Ketols of which 16α-hydroxyestrone and 16 ketoestradiol-17β are the most important compounds. These have been measured in a few diabetic and normal pregnancies and have formed the basis of separate reports.[10,11]

Since estriol represents by far the largest part of the estrogens excreted in the urine in pregnancy—some 75 per cent—it is generally held to be the one most representative of over-all estrogen production. Our results indicate that in half of diabetic pregnancies the urinary excretion of estriol is below the normal range. Taylor has recently reported that estriol excretion in seven diabetics was within the normal range.[12] An occasional patient has an extremely high excretion. In determining if correlations exist between the clinical course of the pregnancies and the estriol values we are first confronted by the problem that there are so

many variables that the small number of patients does not permit firm correlations. There are a number of single interesting observations, but, in a small series, it is impossible to tell whether these are coincidences only or could be substantiated by a larger series. In an effort to find common factors one may easily gloss over individual abnormalities which might have considerable significance.

Two intra-uterine deaths occurred in patients with renal damage. In Case I there was a precipitous drop in estriol excretion. It is our opinion that with the new rapid methods of estimation intra-uterine death could have been foretold and cesarean section would then have become mandatory. In Case II, the failure of the estriol excretion to rise after the thirty-third week probably indicated impending intra-uterine death. A third patient with nephropathy showed a similar pattern of estrogen excretion but went into spontaneous labor at thirty-seven weeks. Although primiparous, she delivered a small healthy infant. It should be stressed that it may be misleading to infer too much from measurements of urinary estrogens in patients with impaired renal function.

The diabetic who had the lowest estrone values on two occasions delivered small babies after forty weeks' gestation. The patient with very high urinary estrone values had similar values on another pregnancy not recorded in this series.

It is our impression that older women and those with long-standing diabetes may have lower levels of estrogen excretion if they have degenerative complications.

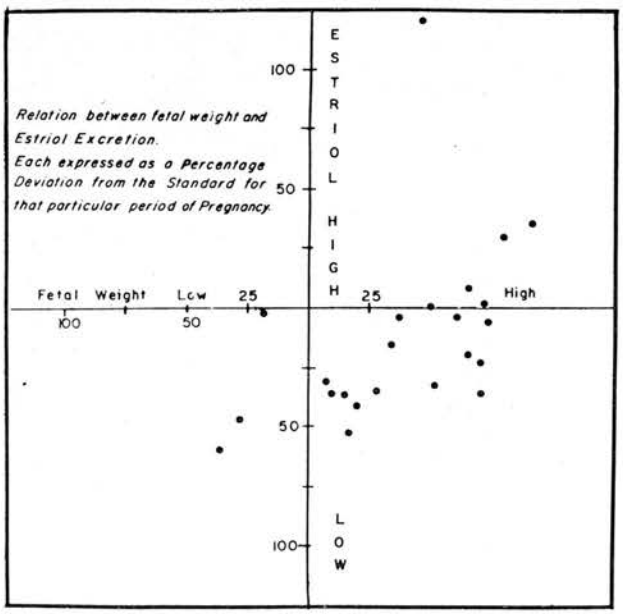

FIGURE 6

We have the impression that women with higher estrogen production have bigger babies (figure 6). The weight of the infant at birth has been compared to the weight for that degree of maturity given by standard tables and is expressed as a percentage deviation. The last urinary estriol excretion before delivery has been compared arbitrarily to the normal for that period of gestation and also expressed as a percentage deviation. Since estriol excretion in diabetics tends to be low, most points are below the horizontal. Since infants of diabetics tend to be big, most of the points are to the right of the vertical, i.e., in the right lower quadrant. The lowest estrogen values and smallest babies coincide and the highest excretions and biggest babies coincide. Most other points would seem in a rough way to lie between. This gives us the impression that there is a correlation between the estrogen excretion and baby size, the women with higher excretion having the bigger babies, and vice versa.

We can show no correlation of estrogens with maternal weight gain, insulin requirement or change in insulin requirement during pregnancy. The influence of diet on estrogen excretion has not been studied. A very much larger series of cases will be required before firm conclusions as to the significance of estrogen excretion studies can be made. Our knowledge of normal estrogen metabolism must also progress. Notwithstanding the interest in the use of estrogens in diabetic pregnancy for the last twenty-five years, progress has been hampered by the difficulties of measurement. In this study the chemical studies were so laborious and time consuming that the results were not of use to the clinician. Within very recent years, relatively simple chemical methods have been described by Ittrich[13,14] for total urinary estrogen measurement. The simpler of these[13] yields values that agree closely with the total of separate estrogen fractions.[10] In analyses of urines that contain sugar, the slightly longer procedure[14] is required. This yields values which may be significantly lower than the true level present.[11] This is probably due, at least partly, to destruction of the labile ring-D Ketols which may contribute substantially to the total, particularly in diabetic pregnancies.

SUMMARY

Urinary estriol has been measured in the second half of twenty-six diabetic pregnancies using Hobkirk's modification of Bauld's method. Half of the values are below the normal range; occasional values are above.

The mean of diabetics is lower than the mean of normal. Most values for estrone and estriol are within the normal range but occasional values are much higher or much lower.

Serial estriol determinations in two pregnancies in diabetics with nephropathy which resulted in intrauterine death are reported.

SUMMARIO IN INTERLINGUA

Estrogenos Urinari in Diabete de Pregnantia: Un Correlation Clinic

Estriol urinari esseva mesurate durante le secunde medietate del pregnantia de vinti-sex diabeticas. Le methodo usate esseva un modification per Hobkirk del methodo de Bauld. Un medietate del valores es infra le limites del area normal. Valores occasional es supra illo. Le valor medie pro diabeticas es plus basse que le valor medie pro normales. In le majoritate del casos, le valores pro estron e estradiol es intra le limites del norma, sed valores occasional es multo plus alte o multo plus basse.

Determinationes serial de estriol in duo pregnante diabeticas con nephropathia, resultante in morte intrauterin, es reportate.

ACKNOWLEDGMENT

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Observations on the Occurrence of Six Estrogen Fractions in Human Pregnancy Urine

I. Normal Pregnancy*†

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ABSTRACT. A chemical method of apparently adequate reliability made it possible to obtain a fairly accurate picture of the qualitative and quantitative aspects of the urinary excretion of 2-methoxyestrone, estrone, ring D α-ketolic estrogens, estriol, 16-*epi*estriol and estradiol-17β (estradiol) in human pregnancy. With the exception of 2-methoxyestrone, these fractions increased during the last 20 weeks of pregnancy. 2-Methoxyestrone behaved in an irregular fashion and was frequently not present in measurable amounts. Further fractionation of the ring D α-ketolic estrogens showed the main constituents to be 16α-hydroxyestrone and 16-ketoestradiol-17β (16-ketoestradiol). During the last four weeks of pregnancy the mean corrected values for the 24-hour excretion of the various fractions in 7 normal pregnancies were: 2-methoxyestrone, 0.17 mg; estrone, 1.4 mg; 16α-hydroxyestrone, 2.9 mg; 16-ketoestradiol, 2.0 mg; estriol, 26.4 mg; 16-*epi*estriol, 1.2 mg; and estradiol, 0.56 mg. The excretion of the 6 fractions taken together agreed closely with total estrogen excretion as measured by the Ittrich technique.

ATTEMPTS at quantitative measurement of urinary estrogens in human pregnancy have been to date almost wholly confined to the three classic compounds, estrone, estradiol-17β (estradiol) and estriol (1, 2). Within the last six years, however, a number of additional estrogen metabolites have been recognized either as metabolites of exogenous steroids labeled with radioactive carbon or as endogenous products excreted during pregnancy. Among the latter are 16-*epi*estriol (3), 2-methoxyestrone (4) and the ring D α-ketols such as 16α-hydroxyestrone (5), 16-ketoestradiol-17β (16-ketoestradiol) (6), and 16β-hydroxyestrone (6). A chemical method for the separation and measurement of six urinary estrogen fractions (7) including estrone, estradiol, estriol, 2-methoxyestrone, ring D α-ketolic estrogens and 16-*epi*estriol has made possible a more complete study of urinary estrogens. The main purpose of the present work was to provide a more complete picture of both the qualitative and quantitative aspects of excretion of these estrogen metabolites during the last twenty weeks of pregnancy. Particular attention was paid to the reliability of the methods employed.

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Materials and Methods

Subjects. Seven pregnant women exhibiting no clinical abnormalities and receiving no medication were studied during the last 20 weeks of their pregnancies. In 4 of the subjects, urinary estrogen fractionation was performed at intervals of 2–4 weeks; in the remaining 3, the analyses were carried out less frequently. Thirty-six fractionations were performed.

Urine collection. Complete 24-hour specimens were collected in polyethylene bottles, without preservative. Enzymic hydrolysis of the conjugated estrogens was commenced as soon as each urine collection was completed. If, after hydrolysis, delay

was necessary prior to subsequent analytical steps, the urine was frozen until needed.

Estrogen separation and measurement. Volumes of from 10 to 20 ml of urine were analyzed in duplicate by the method of Givner *et al.* (7) modified to include the use of smaller reagent and solvent volumes. Hydrolysis of conjugates was achieved by incubating the urine with 500 units of β-glucuronidase per ml and 250 units of sulfatase (enzymes from the digestive tract of the snail *Helix pomatia*)[1] per ml for 24 hours at pH 5.2 and 37°C—a procedure which has proved to be very efficient in this laboratory (8). Following incubation the urine was diluted to 100 ml with water prior to extraction of free estrogens and separation of these by a modified Girard reaction (9). The ketonic and nonketonic fractions were then treated according to Givner *et al.* (7) up to the stage of separation by partition chromatography. Estrogen fractions separated at this stage were 2-methoxyestrone, estrone, ring D α-ketols (as a group), estradiol, 16-*epi*estriol and estriol. Saponification procedures for the further purification of 2-methoxyestrone and estradiol were omitted since these resulted in no particular benefit provided the Celite columns had previously been packed sufficiently firmly to ensure adequate separation of the estrogens from nonspecific chromogens of similar chromatographic mobilities. Spectrophotometric measurement of each estrogen fraction (in terms of the pure standard estrogens concerned, and using 16 α-hydroxyestrone as standard for the ring D α-ketolic fraction) was made by a modified Kober reaction (7) applying the Allen equation to correct for background color (10).

Separation of the components of pooled ring D α-ketolic fractions obtained from a number of late pregnancy urines was achieved by paper chromatography in the system chloroform:formamide (6). Estrogen-containing zones (visualized by the Folin reagent) corresponding in mobility to 16 α-hydroxyestrone, 16-ketoestradiol and (provisionally) 16 β-hydroxyestrone were cut from the chromatograms along with appropriate paper blanks, and eluted with ethanol. The dried eluates were partitioned between ether and water to remove residual formamide, and the ether extracts were dried over anhydrous sodium sulfate prior to colorimetric measurement by the procedure described by Ittrich (11), employing tetrachloroethane as a solvent for the chromogen. Zones corresponding to 16α-hydroxyestrone and 16-ketoestradiol were measured in terms of pure 16α-hydroxyestrone and 16-ketoestradiol respectively. In the absence of a pure sample of 16β-hydroxyestrone, the provisional 16β-hydroxyestrone zone on the paper was measured in terms of 16-ketoestradiol. Corrected optical densities of eluates from the paper blanks were consistently zero, provided previously washed paper was employed for chromatography.

Measurement of total estrogens in pregnancy urine. This was performed in duplicate by the method of Ittrich (11) and involved a direct Kober color reaction on 0.15 ml of urine followed by extraction of the chromogen into a 2% p-nitrophenol solution in tetrachloroethane. Optical density was measured at 506, 538 and 570 mμ and the Allen equation was used to correct for background color. Total estrogen levels were expressed in terms of estriol.

Reliability of the analytical methods. *a) Accuracy.* Sixteen duplicate experiments were performed in which known amounts of pure estrogens (5–25 μg, depending upon the compound concerned) were added to 10 or 20 ml of nonpregnancy urine which had been incubated with the snail enzyme preparation. The described fractionation technique was applied, and the recovery of each estrogen measured. Percentage recoveries (±the standard deviation) were as follows: 2-methoxyestrone, 72 ±6.0; estrone, 85 ±3.7; 16α-hydroxyestrone or 16-ketoestradiol, 64 ±3.6; estradiol 80 ±6.4; and 16-*epi*estriol, 75 ±5.0.

The accuracy of the total estrogen method was determined by measuring the recovery of estrogens added to 0.15 ml of nonpregnancy urine in duplicate analyses. When estriol (1–4 μg) was added, the percentage recovery figures were 95 ±5.9. In three experiments using 4–8 μg of 16α-hydroxyestrone and 5–10 μg of 16-ketoestradiol separately, the values were in the range 93–101% and 83–86% respectively.

b) Precision. Results obtained from the duplicate analysis of a number (>30) of pregnancy urines were employed to calculate the degree of precision obtainable for the measurement of the six estrogen fractions. Table 1 gives a measure of the standard de-

[1] Glusulase is the commercial name for a snail enzyme extract available from Endo Laboratories Inc., New York.

TABLE 1. Precision of the method for measuring 6 estrogen fractions

Estrogen fraction	Range (mg/24 hrs.)	N*	s†
2-Methoxyestrone	0.12– 0.69	37	.044
Estrone	0.35– 0.99	54	.060
	1.00– 2.35	42	.061
Ketols	0.40– 1.99	37	.10
	2.00–10.50	43	.35
Estriol	3.00– 9.99	42	.29
	10.00–29.5	30	.70
16-*Epi*estriol	0.18– 0.49	41	.024
	0.50– 1.30	44	.047
Estradiol	0.12– 0.29	41	.028
	0.30– 0.95	40	.059

* Number of duplicate analyses.
† Measure of standard deviation (for derivation, see text).

viation (s) calculated from the expression $s = \sqrt{\Sigma d^2/2N}$, where d = the difference between duplicate analyses and N = the number of duplicate analyses (12). Similar treatment of the results of total estrogen measurement showed $s = 1.17$ mg per 24 hours.

c) Sensitivity. Because of the elevated urinary excretion of estrogens during the last twenty weeks of pregnancy, the sensitivity of the fractionation procedure was adequate in all cases for the measurement of 5 out of 6 fractions, the exception being 2-methoxyestrone. In the cases in which there was no measurable 2-methoxyestrone, it should be assumed that the 24-hour excretion of this metabolite was less than 80 μg, since this was the approximate sensitivity with which this steroid could be detected under the experimental conditions. Analysis of larger volumes of urine resulted in troublesome background color in the 2-methoxyestrone fraction.

The lower limit for the measurement of total estrogen lay between 1 and 2 mg per 24 hours (depending upon urine volume), *i.e.*, well below the amount present in urine collected during the last twenty weeks of normal pregnancy.

d) Specificity. Some information in support of the specificity of the fractionation technique has already been presented by Givner *et al.* (7). In the present study, the additional paper chromatographic evidence for the identification of the separated estrogen fractions is in general agreement with their data. However, the presence of a second phenolic zone, of greater mobility than 16-*epi*estriol, was repeatedly observed in the fraction associated with the latter compound. This may have been an isomeric triol but it was obviously of much less quantitative significance than 16-*epi*estriol. The 16-*epi*estriol-containing fraction from pregnancy urine consistently gave rise to an extremely rapid development of color in the first stage of the Kober color reaction—an observation in accord with the presence of an estrogen containing *cis*-hydroxyl groups in ring D (3).

Chemical reduction of urinary ring D α-ketolic estrogen fractions with sodium borohydride (5, 13) gave rise to Kober-positive material, 98% of which was found in the estriol and 16-*epi*estriol fractions following column chromatography. Paper chromatography of the reduction products showed a greater amount of estriol than of 16-*epi*estriol; a third very faint and uncharacterized Folin-positive zone, less polar than 16-*epi*estriol, was also observed. Borohydride reduction of the 16-ketoestradiol and 16α-hydroxyestrone fractions separated from a urinary ring D α-ketolic estrogen fraction by paper chromatography yielded respectively 16-*epi*estriol and a mixture of two compounds, the main one of which was estriol and the other possibly 17-*epi*estriol (5). A third small component of the urinary ring D α-ketolic fraction, more polar than 16-ketoestradiol in the solvent system chloroform:formamide, was provisionally considered to be 16β-hydroxyestrone. It was not obtained in sufficient quantity to treat by the reduction technique. These observations showed that the ring D α-ketolic estrogen fraction of the urine in late pregnancy, as

TABLE 2. Urinary excretion (mg/24 hrs.) of 6 estrogen fractions in 4 normal pregnancies

Subject	Estrogen* fraction	Week of pregnancy 20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
	2-Meo-o		0.14			0.17		—		0.19		0.12		0.19		0.14		0.22		0.32		0.19
	O		0.66			0.75		0.57		0.81		0.90		1.0		0.97		1.3		1.5		1.5
T.B.	Ketols		1.6			1.0		1.0		1.7		1.7		1.8		2.2		2.7		—		2.9
	T		4.6			6.5		7.6		9.8		10.0		12.1		12.6		18.3		14.9		16.8
	epi T		0.23			0.33		0.32		0.36		0.36		0.51		0.58		0.72		0.67		0.60
	D		0.25			0.21		0.25		0.25		0.30		0.33		0.38		0.47		0.59		0.37
	2-Meo-o						0			0		0		0			0			0		0
	O						0.85			1.2		1.2		1.4			(0.43)			(0.31)		(0.61)
P.K.	Ketols						1.5			2.4		2.2		2.8			2.2			2.4		4.6
	T						5.9			7.5		9.2		9.5			12.3			15.0		22.0
	epi T						0.33			0.48		0.55		0.56			0.75			0.81		1.2
	D						0.30			0.35		0.36		0.61			0.33			0.32		0.42
	2-Meo-o			0.11				0.10					0.16				0				0	
	O			0.73				0.79					1.0				0.90				1.2	
M.S.	Ketols			1.8				2.6					3.0				4.3				5.9	
	T			9.5				13.3					13.5				17.0				31.2	
	epi T			0.52				0.55					0.59				1.1				1.4	
	D			0.24				0.27					0.37				0.34				0.60	
	2-Meo-o	0				0				0				0				0	0			
	O	0.33				0.52				0.40				0.55				0.59	0.58			
H.J.	Ketols	0.77				1.3				1.7				2.1				2.7	2.5			
	T	5.0				9.9				12.2				16.3				20.6	22.1			
	epi T	0.25				0.43				0.56				0.61				0.82	0.83			
	D	0.19				0.20				0.32				0.37				0.42	0.48			

* 2-Meo-o =2-methoxyestrone; O =estrone; T =estriol; epi T =16-*epi*estriol; D =estradiol.

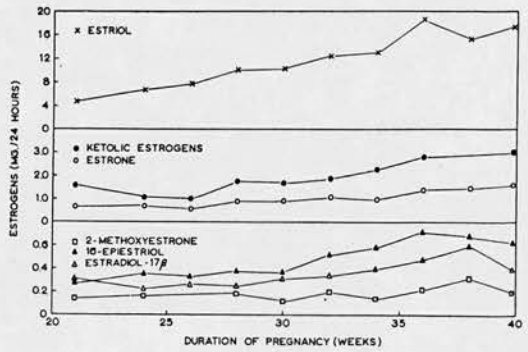

FIG. 1. Excretion of 6 urinary estrogen fractions during the last 20 weeks of pregnancy in Subject T.B. Results are uncorrected for methodological losses.

obtained by the fractionation technique employed, consists mainly of 16α-hydroxyestrone and 16-ketoestradiol.

Results

Pattern of the six estrogen fractions. Figure 1 shows the results obtained in a normal pregnancy in which estrogen fractionations were performed on the urine at intervals of two weeks. There was a general increase in all six fractions measured over the 20-week period. Noteworthy are the low excretion of 2-methoxyestrone, the consistency with which 16-*epi*estriol levels remained higher than those of estradiol, and the fact that ring D α-ketolic estrogen levels were second, in a quantitative sense, to those of estriol. These values were not corrected for methodological losses.

The results obtained in 4 normal pregnancies, uncorrected for experimental losses, are shown in Table 2. In each subject estriol was by far the main urinary metabolite, with the ring D α-ketols second in quantitative importance. The excretion of estradiol was always at least slightly lower than that of 16-*epi*estriol. In Subject M.S., who during late pregnancy excreted considerable amounts of estriol and ring D α-ketols, no 2-methoxyestrone was detectable after about week 31. This was in contrast to Subject T.B. who, during weeks 36–40, excreted 0.19–0.32 mg of 2-methoxyestrone despite the much lower levels of estriol and ring D α-ketols than in Subject M.S. It is also of interest that the urinary levels of estrone and estradiol in these 2 subjects were similar, although the amounts of the 16-oxygenated metabolites (estriol, ring D α-ketols and

TABLE 3. Urinary excretion (mg/24 hrs.) of 6 estrogen fractions in 7 normal pregnancies (values are arithmetic means with ranges in parentheses)

Estrogen* fraction	Period of pregnancy (weeks)			
	20–25	26–30	31-35	36–40
2-Meo-o	0.10 (0–0.19)	0.13 (0–0.51)	0.10 (0–0.35)	0.12 (0–0.33)
O	0.73 (0.33–1.1)	0.98 (0.40–2.0)	1.0 (0.55–1.4)	1.2 (0.58–1.8)
Ketols	1.3 (0.65–1.8)	2.0 (1.0–2.6)	2.4 (0.92–4.3)	3.2 (2.2–5.9)
T	6.5 (3.7–9.9)	10.0 (7.5–13.3)	12.5 (6.7–17.0)	19.8 (14.9–31.2)
epi T	0.34 (0.23–0.52)	0.48 (0.32–0.69)	0.63 (0.36–1.1)	0.88 (0.60–1.4)
D	0.23 (0.16–0.30)	0.36 (0.25–0.47)	0.38 (0.29–0.61)	0.45 (0.32–0.60)

* For key to abbreviations see Table 2.

TABLE 4. Comparison of urinary excretion (mg/24 hrs.) total estrogens (Ittrich method) with the excretion of 6 estrogen fractions in normal pregnancy

Urine No.	Total estrogens, Ittrich method	6 Estrogen fractions (corrected for exptl. losses)		6 Estrogen fractions (uncorrected for exptl. losses)	
		Total	% of Ittrich value	Total	% of Ittrich value
1	25.6	23.8	93	17.6	69
2	51.8	54.9	106	40.4	78
3	13.3	13.8	104	10.2	77
4	25.3	22.1	87	16.4	65
5	10.7	11.5	107	8.6	80
6	11.0	9.9	90	6.5	60
7	17.9	16.6	93	12.3	70
8	18.9	20.4	108	15.1	80
9	20.0	26.9	135	19.9	100
10	33.2	34.0	102	25.1	76
11	20.5	19.5	95	13.4	65
12	9.6	8.0	83	6.0	63
13	15.2	13.1	86	9.9	65
14	30.9	31.3	101	23.3	75
		Mean ±S.D. =99 ±10.1%		Mean ±S.D. =73 ±13.2%	

16-*epi*estriol) were considerably higher in Subject M.S. The onset of slight hypertension in Subject P.K. at about week 34 approximately coincided with a significant drop in urinary estrone excretion—a change which was not shared by the other metabolites. The reason for this is obscure.

Table 3 contains data on the mean values and ranges for the 6 estrogen fractions over various periods during the last twenty weeks of pregnancy in the 7 subjects studied. As before, all results were uncorrected for experimental losses. The ring D α-ketols and 16-*epi*estriol, like estriol, tended to increase to a relatively greater extent than estrone or estradiol as pregnancy progressed. Estriol alone accounted for 73 ± 6.1 (S.D.)% of the total of the six fractions over the last twenty weeks; the corresponding value for the ring D α-ketols was 13 ± 3.1%. Thus these 2 fractions together amounted, on the average, to 86% of this total.

Paper chromatography of mixtures of pure 16α-hydroxyestrone and 16-ketoestradiol, followed by elution and colorimetric measurement, consistently yielded 60–70% recovery. Likewise, fractionation by this technique of 55 μg of a urinary ring D α-ketolic fraction (in terms of 16α-hydroxyestrone) yielded 22 μg of 16α-hydroxyestrone, 15.2 μg of 16-ketoestradiol and (provisionally) 0.8 μg of 16β-hydroxyestrone, *i.e.*, a total recovery of 38 μg or 69%. When these values are used, and the levels of the estrogen fractions in late pregnancy urine are corrected for the average losses known to be incurred during the over-all fractionation procedure, the mean values (weeks 36–40) in Table 3 become (in mg/24 hours): 2-methoxyestrone, 0.17; estrone, 1.4; 16α-hydroxyestrone, 2.9; 16-ketoestradiol, 2.0; 16β-hydroxyestrone, 0.1; estriol, 26.4; 16-*epi*estriol, 1.2; estradiol, 0.56; and total estrogens, 34.7. Taking the highest values found during the same period, the corrected figures are: 2-methoxyestrone, 0.46; estrone, 2.1; 16α-hydroxyestrone, 5.5; 16-ketoestradiol, 3.6; 16β-hydroxyestrone, 0.1–0.2; estriol, 41.7; 16-*epi*estriol, 1.9; estradiol, 0.75; and total estrogens, 56.0.

Total urinary estrogens. Table 4 shows the relationship between the six estrogen fractions added together and the total estrogens as measured by the Ittrich procedure in 14 urines from five

subjects. A close correlation was obtained considering the different methods employed, and it was obvious that the difference between the uncorrected values for the 6 fractions and the Ittrich totals was to a large extent the result of methodological losses associated with the longer fractionation technique.

Discussion

Appraisal of the reliability of the fractionation technique employed would indicate that it has been possible to achieve the main objective of this study, namely, to obtain a fairly accurate picture, qualitatively and quantitatively, of the pattern of six estrogen fractions in normal human pregnancy urine. It should be clearly understood that no cognizance has been taken of a number of other recently discovered estrogen metabolites such as 2-methoxyestriol (14), 18-hydroxyestrone (15) and 2-hydroxyestrone (16) which could perhaps, although probably only to a small extent, contaminate certain of the fractions actually measured.

A problem arose because 2-methoxyestrone was frequently not measurable in pregnancy urines. At an early stage in the study it was thought that this might be due to technical factors such as the occasional occurrence of an undesirable background color in the 2-methoxyestrone fraction (see Methods). Moreover, for some unknown reason, recoveries of this steroid (average = 72%) were considerably lower than the values reported by Givner *et al.* (7). Despite these possible objections, however, it appears that the virtual absence (<80 μg per 24 hours) of 2-methoxyestrone in some pregnancy urines is a true phenomenon. When measurable amounts of the metabolite in question were present, a typical Kober color (λ max. = 550 mμ) was formed, and a discrete Folin-positive zone with the mobility of 2-methoxyestrone could always be observed on paper chromatograms. The irregular occurrence of this compound in these pregnancy urines may have been dependent upon the degree of availability of factors (*e.g.*, tetrahydrofolic acid) which may play a part in the formation of 2-methoxylated estrogen (17).

The qualitative and quantitative patterns of estrone, estradiol and estriol excretion in the present study were similar to those already reported both from this laboratory (2) and elsewhere (1). However, the present results and other data from this laboratory (2) demonstrate estriol levels which, although in the same range as those of other workers, do not approach the highest levels reported by them to occur in late pregnancy (18). It is unlikely that this represents technical error; rather, it is a true variation. The levels of 16-*epi*estriol reported in this paper are consistent with those found by others (19).

The urinary ring D α-ketolic estrogen metabolites are obviously of considerable quantitative importance in human pregnancy. The ratio between 16α-hydroxyestrone and 16-ketoestradiol in the present study was rather close to that found by Layne and Marrian (6) in an isolation experiment. However, the 16β-hydroxyestrone provisionally measured in this laboratory represented a much smaller fraction of the ring D α-ketols than reported by the latter investigators. One possible explanation for this would be the ease with which 16β-hydroxyestrone can be chemically rearranged to yield 16-ketoestradiol. It is interesting that, in general, the 16-oxygenated estrogen metabolites (ring D α-ketols, estriol and 16-*epi*estriol) increase to a greater extent during the later stages of pregnancy than do estrone and estradiol. Moreover, unpublished results from this laboratory have shown that in the nonpregnant state the urinary excretion of

estrone is about equal to that of the ring D α-ketols. These findings are not inconsistent with the view that the ring D α-ketols (probably precursors of estriol and 16-*epi*estriol) may arise in the placenta by biosynthetic routes other than from estrone and estradiol. Such a state of affairs has already been shown to exist for 16α-hydroxyestrone (20). Besides these possibilities, it must not be overlooked that the renal handling of estrogens may play an important part in the urinary pattern (21).

Any consideration of the Ittrich technique for measuring total estrogens must take into account the fact that it measures a number of compounds of different extinction coefficients in terms of a single estrogen (estriol)—a situation resembling that involved in the measurement of total 17-ketosteroids. Because of this factor, a critical comparison between the excretion of total estrogens and the excretion of the 6 fractions is not possible. However, in view of the close similarity of the results obtained by these two types of measurement, it is apparent that the estrogen fractions which were studied must account for practically all the quantitatively important Kober-positive compounds in the urine, and that the Ittrich method yields a satisfactory measure of total estrogens.

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OBSERVATIONS ON THE OCCURRENCE OF SIX ESTROGEN FRACTIONS IN HUMAN PREGNANCY URINE

II. Diabetic Pregnancy

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ABSTRACT. A study was made of the pattern of 6 urinary estrogen fractions, from the twentieth week of pregnancy onward, in women with diabetes mellitus. These fractions were 2-methoxyestrone, estrone, ring D α-ketolic estrogens, estriol, 16-*epi*estriol and estradiol-17β (estradiol). As in normal pregnancy, 2-methoxyestrone behaved irregularly and frequently was not present in measurable amounts, particularly in urines containing large amounts of estriol. The excretion of ring D α-ketolic estrogen frequently was higher than has been found in normal pregnancy, and reached 16.2 mg per 24 hours in one case when corrected for experimental losses. During approximately weeks 35–37 of pregnancy, the mean corrected values (mg per 24 hours) for the various fractions in the urine of the diabetic patients were: 2-methoxyestrone, 0.40; estrone, 1.5; ring D α-ketols, 10.0; estriol, 24.7; 16-*epi*estriol, 1.0; and estradiol, 0.60. The excretion of the 6 fractions taken together and corrected for experimental losses was usually considerably higher than the excretion of total estrogens as measured by the Ittrich technique in the same urines.

AN EARLIER report from this laboratory (1) showed that the relative amounts of estrone, estradiol-17β (estradiol) and estriol excreted in the urine of pregnant diabetics over the last twenty weeks of pregnancy were, in some cases, more subject to variation than in normal pregnancy. It was particularly noticeable that the amount of estriol excreted in the urine of diabetics could be remarkably low. The finding of a number of additional urinary estrogen metabolites in the past six years has prompted further investigation of this subject. Since the excretion pattern of 6 estrogen fractions in normal pregnancy has already been reported from this laboratory (2), it was obviously of interest to extend this study to diabetic pregnancy.

In the present work the 6 urinary fractions investigated were 2-methoxyestrone, estrone, ring D α-ketolic estrogens (mainly 16α-hydroxyestrone and 16-ketoestradiol-17β), estriol, 16-*epi*estriol and estradiol. The opportunity was also taken to compare the values obtained by a technique for measuring total estrogens (3) with the values obtained for the 6 estrogens following fractionation.

Materials and Methods

Subjects. Analyses were performed on the urines of 9 women during 10 pregnancies, beginning from approximately week 20 of pregnancy. In only 3 of these women was it possible to study estrogen excretion beyond week 36 because of termination (artificially or otherwise) of most of the pregnancies at or before this time. The subjects suffered from varying degrees of diabetes mellitus but none was toxemic during the period of study. Except for maintenance insulin, no medication was given. Forty-two estrogen fractionations were performed.

Urine collection. Complete 24-hour volumes were obtained under conditions described earlier (2).

Estrogen separation and measurement. Estrogen fractionation and subsequent estimation were performed exactly as previously described for normal pregnancy urine (2).

Measurement of total estrogens in

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pregnancy urine. It soon became evident that the direct Ittrich technique for measurement of total estrogens (4), involving a direct color reaction in the urine, was unsuitable for sugar-containing urines. This could be attributed to a) a masking color (λ max. = 510 mμ) produced by the effect of hot 65% sulfuric acid on glucose, and b) a simultaneous destruction of estrogen under these conditions. Because of this difficulty, the "indirect" Ittrich technique (3), devised to measure total estrogens in nonpregnancy urines, was adapted to meet the situation. For this purpose, duplicate 0.5-ml or 1.0-ml volumes of urine were diluted to 10 ml with distilled water prior to hydrolysis of the steroids at 100° C for sixty minutes with 15 volumes % of concentrated HCl. This procedure splits estrogen conjugates without causing undue destruction of estrogens by the sugar-acid mixture (5). Following hydrolysis the phenolic fraction was extracted and purified somewhat according to Ittrich (3). The final dried extracts were dissolved in ethanol and suitable aliquots were assayed by the Ittrich modification of the Kober color reaction (3), using tetrachloroethane as the solvent for the chromogen. Optical density was measured at 506, 538 and 570 mμ and corrected by the Allen equation (6). Total estrogen was measured in terms of estriol.

Reliability of the analytical methods. The reliability of the fractionation technique was no different from that already shown to apply in the analysis of normal pregnancy urine (2).

The accuracy of the total estrogen procedure was studied by measuring the recovery of 1–4 μg of estriol after addition to 0.5–1.0 ml of nonpregnancy urine, diluted 1:10 with water, prior to hot acid hydrolysis. Under these conditions, recovery was 94 $\pm$ 4.9 (S.D.) %. When 8 μg of 16α-hydroxyestrone and 10 μg of 16-ketoestradiol were separately added to nonpregnancy urine under the same conditions, recoveries were only 44% and 57% respectively.

The precision of the total estrogen method was obtained by estimating the standard deviation (s) from the equation $s = \sqrt{\Sigma d^2/2N}$, where d = the difference between duplicates in a number of analyses (>30) and N = the number of duplicate analyses (7). In this case, s = 0.55 mg per 24 hours.

The sensitivity of this total estrogen method was adequate for all urines considered in the present study.

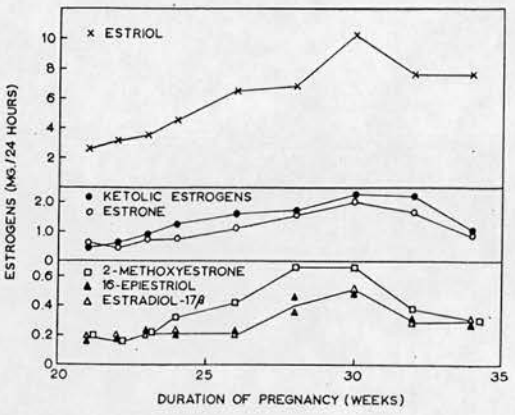

FIG. 1. Excretion of 6 urinary estrogen fractions during the last 14 weeks of pregnancy in Patient M.W. Results are uncorrected for methodological losses.

Results

Pattern of the six estrogen fractions. Figure 1 shows the results, uncorrected for methodological losses, obtained between weeks 21 and 34 in Patient M.W. This particular patient excreted the largest amounts of 2-methoxyestrone that have been measured thus far in this laboratory. It is particularly noteworthy that this high excretion co-existed with a rather low excretion of estriol. All 6 estrogen fractions commenced to diminish at about week 30 of pregnancy in this patient and were quite low just prior to delivery, which occurred at week 35. The reason for this change is not known.

Table 1 contains data on the uncorrected levels of the six urinary estrogen fractions in 4 diabetic women between weeks 20 and 35 of pregnancy. It will be seen that all 4 subjects excreted measurable amounts of 2-methoxyestrone at some stage of the study period, although in 3 (Patients M.B., M.L. and P.B.) this steroid apparently disappeared from the urine (reached a value less than 80 μg per 24 hours), to reappear subsequently in 2 (Patients M.B. and P.B.). At week 26, Patient M.B. excreted 7.9 mg of ring D α-ketols per 24 hours. Although this was not the highest

TABLE 1. Urinary excretion (mg/24 hrs.) of 6 estrogen fractions in 4 diabetic pregnancies

Patient	Estrogen* fraction	Week of pregnancy 20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
	2-Meo-o		0.18	0.16	0.21	0.32		0.41		0.66		0.66		0.38		0.28	
	O		0.46	0.52	0.72	0.73		1.1		1.5		2.0		1.7		0.90	
M.W.	Ketols		0.41	0.62	0.89	1.1		1.6		1.7		2.3		2.2		1.0	
	T		2.6	3.0	3.4	4.5		6.5		6.7		10.1		7.4		7.6	
	epi T		0.16	0.17	0.22	0.18		0.21		0.44		0.45		0.30		0.26	
	D		0.18	0.18	0.18	0.22		0.20		0.35		0.45		0.29		0.30	
	2-Meo-o		0.46		0.55	0.61		0			0		0.45				
	O		1.4		1.4	1.8		2.1			1.2		2.1				
M.B.	Ketols		2.0		2.3	5.8		7.9			4.6		6.1				
	T		5.3		5.8	9.2		11.8			9.2		10.8				
	epi T		0.43		0.32	0.57		0.66			0.51		0.51				
	D		0.53		0.49	1.3		0.96			0.58		0.55				
	2-Meo-o						0.25		0.17		0.15		0		0		0
	O						3.8		3.0		5.1		5.2		3.2		3.5
M.L.	Ketols						3.4		2.2		5.2		6.3		4.8		6.5
	T						11.0		14.3		13.9		14.5		12.5		13.0
	epi T						0.56		0.61		0.61		0.62		0.46		0.59
	D						0.62		0.75		0.79		0.86		0.71		0.52
	2-Meo-o	0		0		0.21			0		0.20	0.32					
	O	0.60		0.62		1.1			1.4		1.4	2.2					
P.B.	Ketols	0.90		0.67		1.4			1.7		1.2	3.9					
	T	4.0		5.6		9.4			10.1		7.8	14.9					
	epi T	0.33		0.61		0.55			1.3		0.94	0.77					
	D	0.14		0.21		0.27			0.36		0.29	0.72					

* 2-Meo-o =2-methoxyestrone; O =estrone; T =estriol; epi T =16-*epi*estriol; D =estradiol.

absolute value found for this fraction, it accounted for 34% of the total of the 5 fractions measured at that time. The ratio of estriol to ring D α-ketols was only 1.5. Patient M.B. also excreted remarkably large amounts of estradiol at weeks 24 and 26 of pregnancy. The urinary estrone level in Patient M.L. reached 5.2 mg per 24 hours, which is well above the normal range. This was the second pregnancy studied in this woman, and it is noteworthy that on the previous occasion (some three years before) the corresponding values were 4.5–6.0 mg per 24 hours during weeks 28–34 of pregnancy (1). It was obvious, therefore, that this phenomenon was reproducible.

Since in most of these diabetic subjects pregnancy terminated some time before term, it was not possible to obtain much data on excretion patterns in late pregnancy. A few isolated results in 3 patients during weeks 35–37 are listed in Table 2. As before, these values were uncorrected for experimental losses. In

TABLE 2. Urinary excretion (mg/24 hrs.) of 6 estrogen fractions during late pregnancy in 3 diabetic women

Patients	Week of pregnancy	Estrogen fraction* 2-Meo-o	O	Ketols	T	epi T	D
S.A.	35	0	2.3	10.5	23.7	1.1	0.56
	36	0	1.7	10.2	23.1	0.86	0.65
M.C.	36	0.68	1.9	4.0	15.4	0.88	0.60
	37	0.53	1.7	5.3	19.8	1.1	0.82
M.K.	36	0	0.37	6.2	29.4	1.0	0.27

* For key to abbreviations see Table 1.

TABLE 3. Urinary excretion (mg/24 hrs.) of 6 estrogen fractions in 10 diabetic pregnancies (values are arithmetic means with ranges in parentheses)

Estrogen* fraction	Period of pregnancy (weeks)			
	20–25	26–30	31–35	36–40
2-Meo-o	0.23 (0–.61)	0.20 (0–.66)	0.10 (0–.45)	0.29 (0–.68)
O†	0.91 (.17–1.8)	1.5 (.64–2.2)	1.4 (.74–2.4)	1.3 (.37–1.9)
Ketols	1.8 (.41–5.8)	3.2 (1.2–7.9)	5.9 (1.0–10.5)	6.5 (4.0–10.5)
T	6.1 (2.6–11.0)	11.5 (6.5–22.0)	14.8 (6.7–24.1)	18.5 (11.2–29.4)
epi T	0.40 (.16–.72)	0.57 (.21–1.3)	0.60 (.26–1.1)	0.78 (.25–1.1)
D	0.36 (.12–1.3)	0.50 (.20–.96)	0.45 (.18–.86)	0.47 (.17–.82)

* For key to abbreviations see Table 1.
† The high estrone values in Patient M.L. (Table 1) were omitted from this table.

Patient M.C. a measurable amount of 2-methoxyestrone was found, but in Patients S.A. and M.K., although considerable amounts of ring D α-ketols and estriol were excreted, no 2-methoxyestrone could be detected. Subject S.A. excreted the largest amount of ring D α-ketols recorded thus far in this laboratory, namely, 10.5 mg per 24 hours (uncorrected) or 16.2 mg per 24 hours when corrected for experimental losses. This accounted for 30% of the five fractions measured in this case.

Table 3 contains data on mean values and ranges (uncorrected for losses) of the six urinary estrogen fractions in the ten pregnancies studied. Over approximately the last 20 weeks of pregnancy, estriol accounted for 64 ± 7.7 (S.D.)% of the sum of the six fractions and the ring D α-ketols accounted for 19 ± 7%. These two fractions, therefore, accounted for about 83% of the total. The 16-oxygenated estrogens showed a relatively greater general increase over the 20-week period than did estrone or estradiol. When the average values for the fractions in weeks 36–40 of pregnancy were corrected for known experimental losses they became (mg per 24 hours): 2-methoxyestrone, 0.40; estrone, 1.5; ring D α-ketols, 10.0; estriol, 24.7; 16-*epi*estriol, 1.0; and estradiol, 0.60.

Total urinary estrogens. In Table 4 the values for total estrogens measured by the Ittrich method are compared with the sum of the values for each of the 6 estrogen fractions measured following the fractionation procedure, in 9 diabetic pregnancy urines. Unlike the picture for normal pregnancy urine (2), the sum of the 6 fractions was often considerably higher than the results obtained by the Ittrich method. Although this may be partly explained by the destruction of ring D α-ketolic estrogens during the preliminary acid hydrolysis in the Ittrich procedure (see Methods), the large difference is difficult to explain adequately.

Discussion

The general pattern of the estrogen metabolites studied in the urine of diabetic pregnant subjects was very similar to that seen in normal pregnancy (1, 2). However, in individual cases there were some interesting features. It has been shown that the excretion of estriol in diabetic pregnancy may be markedly

TABLE 4. Comparison of urinary excretion (mg/24 hrs.) of total estrogens (Ittrich method) with the excretion of 6 estrogen fractions in diabetic pregnancy

Urine No.	Ittrich method	6 Estrogen fractions (corrected for exptl. losses)		6 Estrogen fractions (uncorrected for exptl. losses)	
		Total	% of Ittrich value	Total	% of Ittrich value
1	15.8	22.4	141	16.7	106
2	16.8	20.4	121	15.9	95
3	21.2	20.7	96	15.3	72
4	17.6	26.2	150	19.6	111
5	21.9	27.9	127	21.0	96
6	22.8	34.3	150	24.6	108
7	24.4	37.1	152	27.6	113
8	25.0	29.2	117	21.6	86
9	30.2	32.8	109	24.0	79
		Mean ±S.D. =129 ±20		Mean ±S.D. =96 ±14.7	

lower than in normal pregnancy (1). The results reported here, although not in disagreement with this conclusion, show that estriol excretion in some pregnant diabetics may reach the upper limit of the normal range. The previous finding of a greater variability in the relative proportions of estrogen metabolites in the urine of diabetic pregnancy compared with nondiabetic pregnancy (1) has been substantiated in the present study by the finding of abnormally high estrone excretion in one subject and high estradiol excretion in another. The urinary output of ring D α-ketols in the diabetic subjects rose significantly, and on the average was twice that found earlier for normal pregnancy (2). It would be premature to make a comparison in this latter respect between these groups, but it is interesting that the ring D α-ketolic fraction could make a much higher contribution (up to 34%) to the total urinary estrogens in the diabetics than in the normal women (2). The irregular behavior of 2-methoxyestrone in normal pregnancy (2) was duplicated in the diabetic group. The highest urinary outputs of this steroid in the diabetic subjects tended to be associated with relatively low outputs of the other estrogen fractions. It is suggested that in such cases an increased degree of hydroxylation at the 2-position of the estrogen molecule, followed by methylation, was taking place at the expense of the other fractions.

In the diabetic pregnancy urines the total estrogen levels, as measured by the Ittrich technique, were related much less directly to the sum of the six fractions than in the urine of normal pregnancy (2). It is apparent, therefore, that values for total urinary estrogens by the Ittrich procedure (incorporating hot acid hydrolysis and subsequent purification) must be interpreted with caution.

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2-METHOXYESTRONE AS AN ESTROGEN METABOLITE IN THE HUMAN SUBJECT

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2-Methoxyestrone as an Estrogen Metabolite in the Human Subject[1]

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ABSTRACT. During the last 20–30 weeks of pregnancy, the level of 2-methoxyestrone in the urine of 4 subjects ranged from less than 25 μg/24 hr to about 1 mg/24 hr. The levels fluctuated widely both in any one pregnancy and from one subject to another. There was a great difference in the trend of excretion of this metabolite and that of total estrogens in 2 of the subjects. The behavior of 2-methoxyestrone may be partly due to demethylation to form 2-hydroxy steroids. Some evidence in favor of this was obtained by studying the metabolism of exogenous 2-methoxyestrone in male subjects.

IN PREVIOUS COMMUNICATIONS from this laboratory (1, 2), it was noted that in the pregnant human urinary 2-methoxyestrone exhibited a marked fluctuation throughout any one pregnancy and also varied considerably in amount from one subject to another. This behavior was, in general, quite distinct from that observed for five other urinary estrogen fractions in the same subjects. One limitation of this earlier work resided in the lack of sensitivity of the analytic procedure, so that 80 μg or less of 2-methoxyestrone per 24 hours could not be distinguished from a value of zero. It was thus impossible to ascertain whether or not this metabolite was excreted at all during some pregnancies. In the present study, a rather more sensitive and specific technique was employed to measure urinary 2-methoxyestrone at approximately weekly intervals during the last 25 to 30 weeks of pregnancy. Simultaneous measurement of total urinary estrogens was also performed to compare the excretion with that of 2-methoxyestrone. In order to obtain further information on the problem, an attempt was also made to study the metabolism of exogenous 2-methoxyestrone in human males.

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Materials and Methods

Subjects. Four pregnant women exhibiting no clinical abnormalities and receiving no interfering medication were studied beginning at 12–19 weeks of their pregnancies. Total estrogens and 2-methoxyestrone were measured at intervals of approximately 1 week. Seventeen analyses were performed on Subject M.S., 22 on J.R., 21 on S.S. and 22 on J.C. Two males, aged 47 and 64 yr, both of whom had normal liver function, were employed for the study of exogenous 2-methoxyestrone.

Urine Collection. Complete 24-hr specimens were collected in polyethylene bottles without preservative. Enzymic hydrolysis was commenced as soon as possible after collection. Aliquots were also taken at this time for measurement of total estrogens.

Measurement of 2-Methoxyestrone. Conjugate hydrolysis was performed using the snail enzyme preparation Glusulase[2] under the conditions described earlier (3). Fifty ml volumes of urine were used for each analysis. Free steroids were extracted with ether (4),

[2] Glusulase is the commercial name for a snail enzyme extract available from Endo Laboratories, Inc., New York, N. Y.

and the ether extract was considerably purified by treatment with the NaOH-bicarbonate step described by Bauld (5). The evaporated ether extract was fractionated by a modified Girard reaction (6), and 2-methoxyestrone was separated from the ketonic fraction by partition column chromatography (6). An aliquot of the 2-methoxyestrone fraction was analyzed by the Ittrich colorimetric procedure (7) using tetrachloroethane as solvent. Spectrophotometric readings were made at 536, 568 and 600 mμ and corrected by means of Allen's equation (8). Pure 2-methoxyestrone was used as a standard.

Measurement of Total Estrogens. This was done by the direct Ittrich method (9), as previously described (1). Results were expressed in terms of estriol.

Reliability of the Methods. The above analytic procedure for 2-methoxyestrone resulted in an average increase in recovery of 12% compared with recovery experiments reported earlier (1). Thus, the average recovery figure for the present procedure was 84%, with a standard deviation of 6%. The precision of the method was about the same as with the original technique (1). The sensitivity increased so that the smallest amount which could be distinguished from zero was approximately 25 μg/24 hr. The specificity was improved by the use of the Ittrich colorimetric procedure, which excluded most of the nonspecific chromogens. The reliability of the total estrogen method has already been reported upon (1).

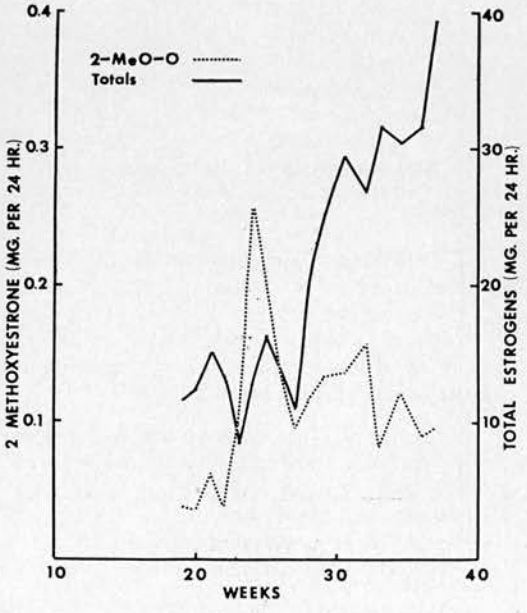

FIG. 1. Excretion of 2-methoxyestrone and total estrogens in Subject M.S. Results are uncorrected for experimental losses.

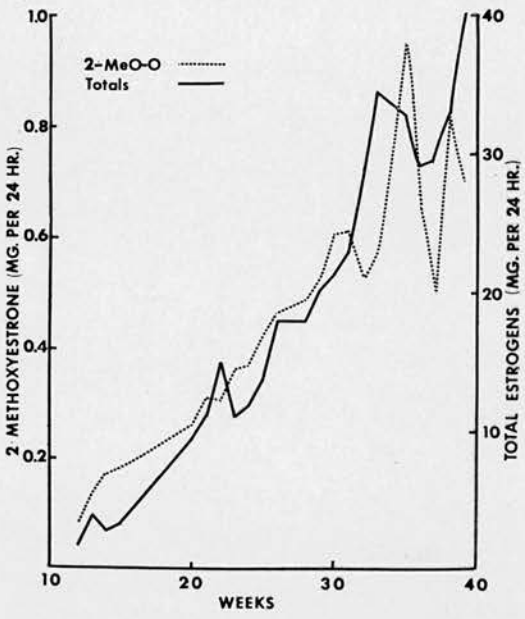

FIG. 2. Excretion of 2-methoxyestrone and total estrogens in Subject J.R. Results are uncorrected for experimental losses.

Metabolism of Exogenous 2-Methoxyestrone. Pure unlabeled 2-methoxyestrone, dissolved in a small volume of propylene glycol, was given intravenously to each of the 2 male subjects. The weights of steroid administered were 500 and 550 μg. Urine was collected in each case for 4 days. Hydrolysis was performed as above, and the total ether fraction was separated into 6 fractions by the procedure of Givner *et al.* (4). Analysis of each fraction was performed by the Ittrich colorimetric method, as described above. In order to obtain further information regarding the identity of 2-methoxyestrone metabolites, thin layer chromatography was employed, as described by Lisboa and Diczfalusy (10), both before and after reduction with sodium borohydride.

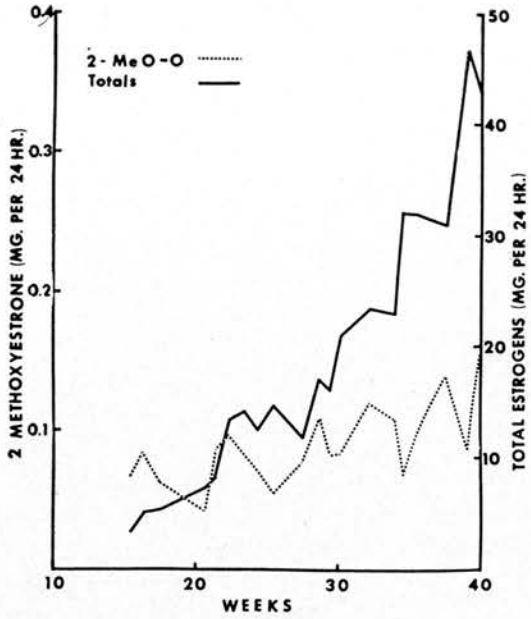

FIG. 3. Excretion of 2-methoxyestrone and total estrogens in Subject S.S. Results are uncorrected for experimental losses.

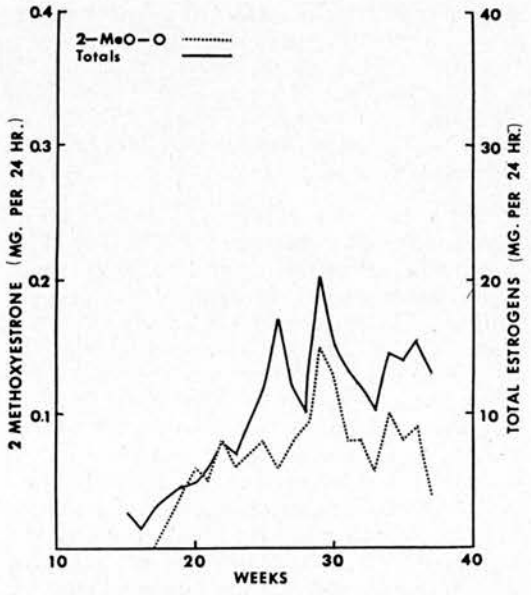

FIG. 4. Excretion of 2-methoxyestrone and total estrogens in Subject J.C. Results are uncorrected for experimental losses.

Results

Fig. 1–4 show the trend in excretion of 2-methoxyestrone and total estrogens in Subjects M.S., J.R., S.S. and J.C., respectively. The results are uncorrected for experimental losses. An abrupt peak in the excretion of 2-methoxyestrone at 24 weeks, with no corresponding pattern of total estrogens, can be seen for M.S. (Fig. 1). Moreover, in this same subject, the 2-methoxyestrone level failed to increase with total estrogens after 28 weeks. A markedly different pattern held for Subject J.R. (Fig. 2), in whom 2-methoxyestrone and total estrogens approximately paralleled each other. Again, in Subject S.S. (Fig. 3), a low and fluctuating 2-methoxyestrone level seemed to bear little or no relation to the total estrogen values. In Subject J.C. (Fig. 4), both 2-methoxyestrone and total estrogens were low, the patterns exhibiting a fairly parallel relationship.

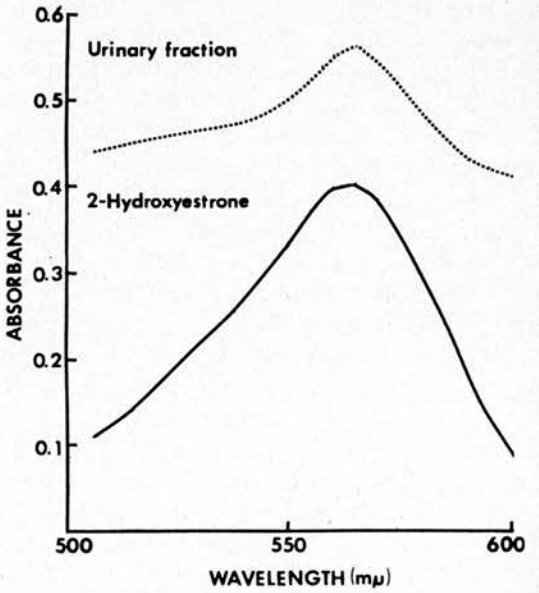

FIG. 5. Spectrophotometric curves of the Ittrich chromogen formed by the 2-hydroxyestrone-like fraction from male urine following injection of 2-methoxyestrone, and of authentic 2-hydroxyestrone.

Except for the first three urines from Subject J.C., measurable 2-methoxyestrone was present in all urines analyzed. The levels of 2-methoxyestrone in these four subjects are summarized in Table 1. As before, no correction has been made for experimental losses.

Following the administration of 2-methoxyestrone to the male subjects, no 2-methoxyestrone was detectable in the urine of one, while in the other case 34 μg, or 6% of the dose, was excreted in four days. In both subjects, material giving a positive Ittrich color reaction occurred in the fractions having the approximate chromatographic mobility of the ring D-α-ketols (6). An absorption peak was observed at about 565 mμ, as for authentic 2-hydroxyestrone (Fig. 5). Thin layer chromatography on silica gel G in the system ethyl acetate:cyclohexane:ethanol (45:45:10) (10), followed by spraying with Folin and Ciocalteu's reagent, showed the urinary fraction to have an R_F value of 0.57 (R_F of 2-hydroxyestrone = 0.57). After borohydride reduction, two spots were visible on the chromatogram, a main one of R_F 0.47 (R_F of 2-hydroxyestradiol-17β = 0.47) and one of lesser intensity with an R_F of 0.57. This suggested the presence of two phenolic compounds, or incomplete reduction. The study was hampered by the presence of a yellow contaminant which was difficult to remove. This, or partial breakdown of the labile 2-hydroxy steroid, may have been responsible for the streaking which occurred on some of the thin layer chromatograms. The 2-hydroxyestrone-like compound appeared to account for some 14% (uncorrected for experimental losses) of the 2-methoxyestrone dose.

TABLE 1. Urinary 2-methoxyestrone levels (mg/24 hr) in four normal pregnancies*

Period of pregnancy (weeks)	No. of analyses	Mean 2-methoxyestrone level	Range of 2-methoxyestrone levels
10–14	3	0.10	0.07–0.14
15–19	10	0.06	0† –0.18
20–24	18	0.13	0.03–0.36
25–29	18	0.18	0.06–0.49
30–34	18	0.23	0.06–0.61
35+	14	0.32	0.04–0.95

* Figures are uncorrected for experimental losses.

† Zero indicates less than 25 μg/24 hr.

Discussion

The data in this communication bear out earlier reports (1, 2) that 2-methoxyestrone is excreted in the urine of pregnant women in a pattern which may appear quite unrelated to the trend in excretion of total estrogens. It is likely that 2-methoxyestrone is always excreted during pregnancy, although often in minute amounts. One possible reason for the behavior of this metabolite could be a variable degree of demethylation with the formation of 2-hydroxy compounds. This has already been demonstrated by Brown (11) for the 3-methyl ethers of estriol, estradiol-17β and estrone. The identity of 2-hydroxyestrone in the urine following 2-methoxyestrone injection has not been proved beyond doubt in the present work, but the supporting evidence is good. Recent work by Fishman *et al.* (12) indicates the thyroid to be an important factor in the conversion of exogenous estrone or estradiol-17β to estriol, on the one hand, and to 2-methoxyestrone on the other. It is not known at present whether or not thyroid function may be responsible for the urinary patterns of 2-methoxyestrone seen in the pregnant subject.

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Contribution of Estriol to Total Urinary Estrogens during Pregnancy

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Estriol accounts for an average of 74% of the principal Kober-positive steroids in the urine of 13 women during uncomplicated pregnancies. This proportion is independent of the stage of pregnancy, from 20 weeks until term. The average ratio of estriol to ring-D α-ketols (mainly 16α-hydroxyestrone and 16-ketoestradiol-17β) is about 5:1 over the same period. In 17 pregnant women with diabetes, urinary estriol averaged 63% of the "total steroids" during a similar period, and the average ratio of estriol to ring-D α-ketols was 3:1. The quantitative importance of these ketolic steroids may introduce a problem in deciding whether to measure estriol or "total estrogens" when evaluating fetal viability in complicated pregnancies.

MEASUREMENT OF URINARY estriol [estra-1,3,5(10)-triene-3,16α,17β-triol] appears to be a useful index to viability of the human fetus (*1–3*). It has been shown that the fetoplacental unit is the main site of estriol synthesis during pregnancy (*4–6*).

Many methods have been published for measuring estriol (*7–9*) and "total estrogens" (*10–12*) in the urine during pregnancy. Although estriol is almost certainly the main single component of the total estrogens in pregnancy, the quantitative relationships are not particularly clear. Confusion has sometimes resulted from the use of the term "total estrogens" to denote the "classical" compounds: estrone [3-hydroxyestra-1,3,5(10)-trien-17-one], estradiol-17β [estra-1,3,5(10)-triene-3,17β-diol], and estriol. It is now known that several other phenolic steroid metabolites contribute significantly to total urinary estrogens (*13*).

Our paper establishes the contribution of estriol to total estrogen excretion and supplements data already obtained in this laboratory (*14, 15*). In view of the interest in the urinary excretion of estriol and total estrogens during pregnancy complicated by diabetes (*16–18*), some comparison is given for normal and diabetic women during their pregnancies.

Materials and Methods

Total 24-h urines were collected without preservative in polyethylene bottles and, unless analyzed immediately, were stored at −15°C until processed.

Reagents

All reagents and organic solvents were purified where necessary by published procedures (*19, 20*).

"Glusulase," a molluscan enzyme preparation containing β-glucuronidase (β-D-glucuronide glucuronohydrolase, EC 3.2.1.31), and sulfatase was purchased from Endo Labs., Inc., Garden City, N. Y. A bacterial β-glucuronidase preparation was purchased from Sigma Chemical Co., St. Louis, Mo.

Crystalline steroids (Mann Research Labs., Inc., N. Y.) were checked for purity by thin-layer chromatography and melting point determination.

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Analytical Methods

Suitable volumes of urine (usually 50 ml) were analyzed by a modification (*21*) of a procedure published elsewhere (*14*). In some instances, "total conjugated" estrogens were hydrolyzed by Glusulase (*22*) while in others steroid glucosiduronates were hydrolyzed by bacterial β-glucuronidase (*21*) and the residual conjugates (mainly sulfates) were cleaved by solvolysis in ethyl acetate and H_2SO_4 (*23*). The various urinary estrogen fractions were purified and separated by solvent partition, Girard separation, and partition chromatography on a Celite column.

The ring-D α-ketolic fraction, consisting mainly of 16α-hydroxyestrone [3,16α-dihydroxyestra-1,3,-5-(10)-trien-17-one] and 16-ketoestradiol-17β[3,-17β-dihydroxyestra-1,3,5(10)-trien-16-one], together with some 16β-hydroxyestrone[3,16β-dihydroxy estra-1,3,5(10)-trien-17-one], was measured by two methods. First, the portion of the column eluate that contained the latter fraction was measured spectrophotometrically in terms of 16α-hydroxyestrone (*14*). Secondly, the fraction was chemically reduced with $NaBH_4$, 16α-hydroxyestrone being converted mainly to estriol (*24*) and 16-ketoestradiol-17β (+16β-hydroxyestrone) to 16-epiestriol [estra-1,3,5(10)-triene-3,16β,17β-triol] (*24*). The two steroidal triols were then separated on Celite and measured individually (*24*). For our purposes these were added to yield "total ring-D α-ketols." The spectrophotometric procedure of Ittrich (*10*) was used throughout, except that tetrachloroethane was used as the solvent for extraction of the chromogen (*20*). Absorbance was measured at 506, 538, and 570 nm in each case and corrected by the method of Allen (*25*). The corrected absorbance was related to the value obtained from known amounts of the appropriate standards. Supporting evidence for the identity of various fractions from urine was obtained by applying aliquots to thin (0.25 mm) layers of Silica Gel H on glass plates, which were then developed in suitable solvent systems (*26*). Spots on the plates were made visible either by spraying with diazotized sulfanilic acid or by spraying with a mixture of 2 ml of H_2SO_4 per 100 ml of ethanol and then heating the plates at 110°C. Mobilities of the various fractions were compared with those of the appropriate pure steroids. Values given in the tables below refer to fractions with R_f values corresponding to the standard steroids in question.

To measure recovery, known amounts of pure steroids, in the approximate proportions expected in pregnancy (*14*), were added to nonpregnancy urine and taken through the experimental procedure. The results were similar to those already published (*14*) but, when the ring-D α-ketolic fraction was reduced, with subsequent extraction and chromatography, about 10% less of it was recovered than by direct spectrophotometry. The average recoveries were estrone, 81%; estradiol-17β, 83%; estriol, 81%; 16-epiestriol, 84%; ring-D α-ketols (direct method), 65%; and ring-D α-ketols (reduction method), 55%. All of the values reported below have been corrected for these average recoveries.

Results

Note that the excretion given for any one steroid represents "total conjugated forms" of that compound.

Table 1 contains data on five fractions from urine obtained during 13 uncomplicated pregnancies (51 analyses). The results are subdivided into four time intervals, 20–24, 25–29, 30–34, and 35 or more weeks of pregnancy. Estriol averaged 74% of the total estrogens measured during each interval. Ring-D α-ketols amounted to about 20% of the estriol concentrations.

Table 2 shows similar data for 17 diabetic

Table 1. Amount of Five Estrogen Fractions in the Urine of 13 Women during Uncomplicated Pregnancies

Weeks pregnant	No. analyses	Estrogen fraction[a] (mg/24 h)						Ratio of E_3 to ring-D α-ketols
		E_1	E_2	Ring-D α-ketols	E_3	16epiE_3	% E_3	
20–24	9	0.90[b]	0.26	1.7	8.8	0.42	73	5.2
		(0.39–1.5)	(0.16–0.34)	(0.42–2.8)	(3.0–13.1)	(0.10–0.69)	(61–79)	(2.5–9.4)
25–29	11	0.98	0.34	2.4	11.6	0.52	73	4.8
		(0.32–1.5)	(0.21–0.44)	(0.99–4.0)	(5.0–17.7)	(0.31–0.75)	(61–81)	(2.6–8.5)
30–34	12	1.4	0.48	3.2	16.3	0.77	73	5.1
		(0.65–2.4)	(0.34–0.76)	(1.4–4.7)	(8.9–21.7)	(0.48–1.1)	(62–81)	(2.7–8.6)
35+	19	1.3	0.46	4.6	24.0	1.1	76	5.2
		(0.21–3.0)	(0.11–0.74)	(1.5–9.1)	(8.7–41.7)	(0.37–1.9)	(67–89)	(3.3–11.1)

[a] E_1, is estrone; E_2, estradiol-17β; E_3, estriol; 16epiE_3, 16epiestriol.
[b] Average, range in parentheses.

Table 2. Amount of Five Estrogen Fractions in the Urine of 17 Diabetic Women during Pregnancy

Weeks pregnant	No. analyses	Estrogen fraction[a] (mg/24 h) E_1	E_2	Ring-D α-ketols	E_3	16epiE_3	% E_3	Ratio of E_3 to ring-D α-ketols
20–24	14	1.4[b]	0.50	2.5	7.9	0.50	62	3.2
		(0.20–3.1)	(0.15–1.6)	(0.63–9.0)	(3.4–12.5)	(0.21–0.95)	(46–76)	(1.3–7.3)
25–29	14	2.1	0.63	4.7	14.3	0.90	63	3.0
		(0.75–5.9)	(0.23–1.2)	(0.88–12.2)	(5.5–29.5)	(0.17–1.8)	(51–74)	(1.5–6.7)
30–34	16	2.1	0.52	6.5	16.7	0.71	63	2.6
		(0.87–6.1)	(0.13–1.1)	(1.4–16.7)	(5.5–32.1)	(0.23–1.2)	(52–82)	(1.8–7.5)
35+	10	2.3	0.57	9.1	24.8	1.0	66	2.7
		(0.43–6.0)	(0.21–1.0)	(4.6–16.2)	(11.0–39.1)	(0.33–1.5)	(53–78)	(1.5–4.7)

[a] E_1 is estrone; E_2, estradiol-17β; E_3, estriol; 16epiE_3, 16epiestriol.
[b] Average, range in parentheses.

Table 3. Excretion of Estrone and Ring-D α-Ketols by Normal and Diabetic Women during Pregnancy

Weeks pregnant	Estrone		Ring-D α-ketols	
	Normal	Diabetic	Normal	Diabetic
20–24	0.90 ± 0.31[a]	1.4 ± 0.85	1.7 ± 0.8	2.5 ± 2.1
25–29	0.98 ± 0.38	2.1 ± 1.4	2.4 ± 1.1	4.7 ± 3.4
30–34	1.4 ± 0.55	2.1 ± 1.4	3.2 ± 0.9	6.5 ± 4.9
35+	1.3 ± 0.79	2.3 ± 1.8	4.6 ± 1.9	9.1 ± 4.1

[a] Mean ± SD.

women during their pregnancies (54 analyses). Estriol averaged 63% of the total and, once more, did not change markedly between 20 weeks and term, although individual values varied considerably. The ratio of estriol to ring-D α-ketols averaged about 3:1. Once again, however, there was a scatter of individual values, reaching as low as 1:3:1.

Ring-D α-ketols averaged higher in the diabetic state than in normal pregnancy, a finding reflected in the ratio of the estriol fraction to ring-D α-ketols. Estrone levels also appear higher in the diabetics. However, none of these differences was statistically significant (Table 3).

No particularly low estriol levels were encountered in the diabetic group, a finding perhaps attributable to the fact that no patient showed signs of intra-uterine death during the period of investigation.

Discussion

The steroids we measured are by no means the only estrogen metabolites present in human pregnancy urines, but they represent the principal steroids showing a strong extinction at 538 nm in the Ittrich modification of the Kober color reaction. It has already been shown that the fractions measured by our analytical procedure compare well as a whole with values obtained by the direct Ittrich method for total estrogens (*14*).

Our data show that between 20 weeks and term, estriol is a fairly constant fraction of the total estrogens. This might suggest that it is immaterial clinically whether one measures estriol or total estrogens. However, we show here that as little as 61% and 46% of the total estrogens measured may be estriol in uncomplicated pregnancies and those with concurrent diabetes, respectively. The greater part of the remainder is ring-D α-ketols. Cohen recently claimed that even smaller proportions of estriol may be found in some toxemic pregnancies (*27*).

The rationale for measuring urinary estriol is that during the second half of pregnancy, about 90% of it originates in the fetus (*6*) and thus it may be useful as an index to fetal viability. Recent work, however, suggests that the ring-D α-ketols, particularly 16α-hydroxyestrone, present in pregnancy urine, originate from the mother rather than the fetus (*21*, *28*). For this reason it may be argued that the determination of the amount of estriol in urine would be more valuable than determination of total estrogens. Cohen suggests the opposite, because estriol may sometimes be a very low fraction of the total (*27*); therefore it might not accurately reflect the total output of steroid by the fetus. The answer to this question may depend on the origin of the urinary ring-D α-ketols, particularly in a condition such as pregnancy complicated by diabetes where these

steroids appear to be of considerable quantitative significance. Cohen also presented indirect evidence for the presence of high levels of these latter steroids in the total estrogen of diabetic pregnancy urines (*27*).

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Specific Activities of Seven Urinary Metabolites of Estradiol-17β-6, 7-^{3}H in Pregnant Women

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ABSTRACT. Estradiol-17β-6,7-^{3}H was injected into 4 women in late pregnancy (33 weeks-term) and 7 labeled metabolites were purified from the urine. These were acetylated with acetic anhydride-1-^{14}C and the acetates were purified by repeated crystallization with unlabeled carrier to constant isotope ratio. The specific activities of the ^{3}H-labeled metabolites were calculated from the ^{3}H:^{14}C ratios and were found to be (cpm/μg): estrone 38–65, estradiol-17β 45–77, 16α-hydroxyestrone 24–44, 16-ketoestradiol-17β 25–36, 16-epiestriol 22–36, estriol 6–8. That of 2-methoxyestrone was not similar to that of estriol. These results may indicate that the endogenous 16-oxygenated metabolites other than estriol are mainly maternal conversion products of secreted estrone or estradiol-17β, with perhaps some part arising through conversion in a fetal compartment. (*J Clin Endocr* **26:** 625, 1966)

IT IS NOW well established that, following injection of labeled estrone or estradiol-17β (hereafter referred to as estradiol) to pregnant women, the specific activity of urinary estriol is markedly lower than that of either estrone or estradiol (1, 2), the two latter having approximately equal values. Such a difference is not so apparent in the nonpregnant subject. These findings have been interpreted as indicating the production of considerable quantities of estriol, by the fetoplacental unit, through a biosynthetic pathway which may be at least partly independent of estrone and estradiol metabolism (3, 4). Thus, placental aromatization of a 16-oxygenated C_{19} neutral steroid (5) could be responsible for the formation of the excess estriol which dilutes the urinary estriol formed by peripheral metabolism of estrone or estradiol in maternal tissues. However, it should also be pointed out that fetal liver has been shown to be an active site of production of 16-oxygenated estrogens from estradiol (6).

The above information prompted us to investigate the specific activities of a number of additional urinary metabolites of injected estradiol-6,7-^{3}H in the human in late pregnancy. Of particular interest is the compound 16α-hydroxyestrone,[1] known to be a normal urinary metabolite of some quantitative significance in pregnancy (7–10) and a good exogenous precursor of urinary estriol *in vivo* in the nonpregnant human (11, 12). If this steroid, as it appears in pregnancy urine, were mainly derived from a fetoplacental source, where it may be a precursor of estriol, it might be expected to have a specific activity similar to that of the triol following labeled estradiol injection. This has now been studied, as have the specific activities of two additional 16-oxygenated metabolites, namely, 16-ketoestradiol-17β (hereafter referred to

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[1] The following abbreviations and trivial names are used in the text: 2-MeE$_1$ = 2-methoxyestrone [2-methoxy-3-hydroxy-1,3,5(10)-estratrien-17-one]; 16α-OHE$_1$ = 16α-hydroxyestrone [3,16α-dihydroxy-1,3,5(10)-estratrien-17-one]; 16β-OHE$_1$ = 16β-hydroxyestrone [3,16β-dihydroxy-1,3,5(10)-estratrien-17-one]; 16-KE$_2$ = 16-ketoestradiol-17β [3,17β-dihydroxy-1,3,5(10)-estratrien-16-one]; ring Dα-ketols = 16α-OHE$_1$ + 16β-OHE$_1$ + 16-KE$_2$; 16-epiE$_3$ = 16-epiestriol [3,16β,17β-trihydroxy-1,3,5(10)-estratriene].

as 16-ketoestradiol) and 16-epiestriol, together with that of 2-methoxyestrone.

Materials and Methods

Chemicals. Estradiol-17β-6,7-^{3}H of specific activity 40 μc/μg was purchased from Merck, Sharp & Dohme Ltd. Following celite column partition (13) the peak tubes were collected and a portion of the material (551,000 cpm) was diluted with 30.8 mg of pure unlabeled estradiol. After crystallization from methanol the crystals and mother liquor had equal specific activities of 17,900 cpm/mg, establishing the radiochemical purity of the material. Acetic anhydride-1-^{14}C of specific activity 10 μc/mg was purchased from New England Nuclear Corp. Unlabeled estrone acetate and estradiol diacetate for use as carriers were purchased from Mann Research Laboratories, Inc., New York. The monoacetate of 2-methoxyestrone was prepared in the laboratory from free steroid purchased from Organon Laboratories Ltd., as was the triacetate of 16-epiestriol. The triacetate of estriol was prepared in the laboratory using estriol obtained from Parke, Davis & Co. Acetylation was performed overnight at room temperature in a pyridine-acetic anhydride (unlabeled) mixture under anhydrous conditions. The residue was crystallized several times from methanol-water mixtures. In all cases the melting points agreed closely with the theoretical. Unlabeled 16α-hydroxyestrone,[2] 16-ketoestradiol (Steraloids Inc., Pawling, N.Y.) and 16β-hydroxyestrone, prepared by hydrolysis (7) of the diacetate (Southeastern Biochemicals Inc., Morristown, Tenn.), were used as paper chromatographic standards. Desoxycorticosterone, for use as a standard in the labeled acetylation procedure, was purchased from Mann Research Laboratories, Inc. All other reagents and solvents were purified where necessary by standard procedures.

Injection of Subjects. Four young pregnant women (LU, SA, SH, IS) between 33 weeks and term were injected via an arm vein with about 7 μc of estradiol-6,7-^{3}H in 10 ml of 10% ethanol in saline. Vials and syringes were washed following injection and the radioactivity remaining was measured to correct for losses. This amounted to less than 2% of the dose. Urine was collected for 96 hr, pooled from each individual and frozen in polyethylene containers until required.

[2] Donated by Dr. A. E. Kellie, Courtauld Institute of Biochemistry, Middlesex Hospital, London, England.

Extraction and Separation. Suitable aliquots of the pooled urines (approx $\frac{1}{2}$) were incubated with bacterial β-glucuronidase (14), the free estrogens (G fraction) were extracted with ether, and the residual urine was subjected to solvolysis (15) to split estrogen sulfates (S fraction). From this point until otherwise indicated, G and S fractions were kept separate. Each was subjected to a modified Girard separation (13). The nonketonic fraction so obtained was further purified by partition between toluene and N NaOH prior to treatment of the phenolic extract by reflux with alkali and re-extraction as described elsewhere (16). The main components of the ketonic and nonketonic fractions were separated by celite column partition chromatography in hexane:benzene/methanol:H_2O systems (13). The main nonketonic metabolites, estradiol, 16-epiestriol and estriol, were in some cases further purified by thin layer chromatography (17) prior to acetylation. The column fraction containing 2-methoxyestrone was further purified by partition between hexane:benzene (3:1) and N NaOH with re-extraction from the aqueous phase following pH adjustment. The estrone fraction was similarly purified using toluene and N NaOH. The ring D α-ketolic fraction was separated into 16β-hydroxyestrone, 16α-hydroxyestrone and 16-ketoestradiol by chromatography for 4 days at room temperature (23 C) on Whatman No. 42 paper against appropriate standards in the solvent system toluene:propylene glycol. Under such conditions these 3 compounds migrate approximately 13, 23 and 35 cm, respectively, from the starting line. Areas corresponding to each compound were cut from the chromatograms and eluted with ethyl acetate. Residual propylene glycol was removed by water washing and the ethyl acetate extracts were evaporated. Because of a lack of crystalline 16α-hydroxyestrone to use as carrier, the separated ring D α-ketols were reduced with sodium borohydride to the corresponding isomeric triols (14). In this way labeled estriol was obtained from urinary 16α-hydroxyestrone and labeled 16-epiestriol from 16-ketoestradiol, and the triols were purified as described below.

Acetylation with Acetic Anhydride-1-^{14}C. The separated estrogen fractions, after drying for at least 16 hr in a desiccator, were acetylated overnight in 0.2 ml of anhydrous pyridine with 0.1 ml of acetic anhydride-1-^{14}C diluted with fresh unlabeled acetic anhydride to give a specific activity so that the final ^{3}H:^{14}C ratios of the acetylated steroids lay between 3 and 10. The acetylation mixtures were partitioned be-

TABLE 1. Pattern of urinary radioactivity (% of injected dose) following chromatography in pregnant women and males receiving estradiol-6,7-^{3}H

Fraction*	Subjects									
	LU		SA		SH		IS		Males†	
	G‡	S‡	G	S	G	S	G	S	G	S
2-MeE$_1$	0.3	0.1	0.4	<0.1	0.8	0.3	0.2	<0.1	1.6	0.5
E$_1$	4.1	1.2	3.3	0.3	4.7	0.7	4.1	2.5	8.0	2.0
E$_2$	0.7	0.2	0.7	<0.1	0.6	<0.1	0.6	0.2	3.4	0.4
ring D α-ketols	4.6	1.4	4.6	0.9	5.5	1.1	7.5	1.9	6.2	0.6
16-epiE$_3$	1.4	0.1	1.1	0.2	1.0	0.2	1.0	<0.1	1.4	0.2
E$_3$	9.4	0.6	6.5	0.2	4.5	0.4	4.6	0.3	5.8	0.4

* See footnote 1 for abbreviations.
† Average of 4 experiments (Hobkirk, unpublished observations).
‡ G = hydrolyzed by β-glucuronidase; S = split by solvolysis.

tween diethyl ether and weak aqueous acid, after which the ether extracts were dried and evaporated. Unlabeled carrier steroid acetates were added in suitable amount (15 mg upward) to each fraction and each was crystallized from methanol-water mixtures until, as far as possible, ^{3}H : ^{14}C ratios of successive crystals differed by no more than 10% and until crystals and corresponding mother liquors showed similar agreement. The specific activities (cpm/μg) of the isolated ^{3}H-labeled metabolites were calculated from the equation:

$$\frac{\text{cpm } ^3\text{H}}{\text{cpm } ^{14}\text{C}} \times \frac{n}{10^3} \times SA_{DOCA} \times \frac{M}{N} \qquad (2)$$

where n = number of acetyl groups introduced into the molecule, SA_{DOCA} = specific activity (cpm/mg) of desoxycorticosterone acetate acetylated by the same sample of acetic anhydride-1-^{14}C and M and N are, respectively, the molecular weights of DOCA and the free estrogen.

All radioactive counting was performed on a Nuclear-Chicago Model 6725 liquid scintillation spectrometer using the formula of Okita *et al.* (18) to calculate the ^{3}H and ^{14}C counts. Efficiencies of counting for these 2 isotopes were, respectively, approximately 25 and 60%. The phosphor used consisted of toluene containing 0.3% of 2,5-triphenyloxazole and 0.01% of 1,4-bis-2-(5-phenyloxazolyl)-benzene.

Results

Table 1 contains data on the urinary radioactivity in the column fractions collected. The fractions containing estrone, ring D α-ketols and estriol contained most of the radioactivity. In most cases some activity was found in the S fractions but this was much less than that in the G fractions. While these urinary patterns should not be examined too critically, since they only represent a general picture of radioactivity distribution, they are not dissimilar to those seen following administration of estradiol-6,7-^{3}H to male subjects (Table 1). Preliminary specific activity measurements on the column eluates, using the spectrophotometric Ittrich modification of the Kober reaction (19) as the means of weight determination, showed no obvious difference between G and S fractions for any one steroid. For further analysis, therefore, G and S fractions were combined for each compound.

Table 2 shows the result of separation of the individual components of the ring D α-ketolic fraction. 16α-Hydroxyestrone was

TABLE 2. Radioactivity (% of dose) associated with separated ring D α-ketols from urine of pregnant women injected with estradiol-6,7-^{3}H*,†

Fraction‡	Subjects			
	LU	SA	SH	IS
16β-OHE$_1$	0.3	—	—	—
16α-OHE$_1$	3.7	1.4	3.3	6.3
16-KE$_2$	1.3	0.9	0.6	1.8

* Each fraction is corrected for a recovery of 70% during paper chromatography.
† G (β-glucuronidase hydrolyzable) and S (split by solvolysis) fractions combined prior to paper chromatography.
‡ See footnote 1 for abbreviations.

TABLE 3. Specific activities (cpm/μg) of urinary metabolites of injected estradiol-6,7-^{3}H in subject LU calculated from successive crystallizations*

Metabolite†	Crystals 1	Crystals 2	Crystals 3	Crystals 4	ML 4	Crystals 5	ML 5
2-MeE$_1$	40	47	45	—	—	—	—
E$_1$	37	43	43	40	—	—	—
E$_2$	—	46	47	45	—	45	47
16α-OHE$_1$	28	28	29	27	29	—	—
16-KE$_2$	31	28	lost	28	26	—	—
16-epiE$_3$	24	25	28	28	25	—	—
E$_3$	9	8	8	8	8	—	—

* Values are based on a dose of 5×10^6 cpm and are rounded off to the nearest whole number.
† See footnote 1 for abbreviations.

the major compound present in each case. In only one experiment was significant radioactivity found in the 16β-hydroxyestrone fraction. The results in Table 2 have been corrected for a known recovery of 70% of pure 16α-hydroxyestrone and 16-ketoestradiol during paper chromatography, so as to make them more comparable with the values of the other steroids which had not been subjected to this process at this stage. Even this correction, however, did not invariably result in close agreement with the radioactivity excreted in the ring D α-ketolic fraction from the column (Table 1). This may be due to removal of other labeled metabolites during paper chromatography.

Tables 3–6 contain data on the specific activities of the ^{3}H-labeled urinary metabolites measured by the double isotope method. The results in each table are corrected to a common injected dose of 5×10^6 cpm of estradiol-6,7-^{3}H and are rounded off to the nearest whole number. The specific activity of estriol is much lower than that of estrone or estradiol, confirming the findings of others. The three other 16-oxygenated metabolites, 16α-hydroxyestrone, 16-ketoestradiol and 16-epiestriol, have values which are similar to each other and which resemble those of estrone and estradiol much more closely than they do that of estriol. The specific activity of 2-methoxyestrone was also very different from that of estriol, although in no case was a mother liquor obtained which corresponded well with the crystals.

Discussion

The results obtained in the present work for estrone, estradiol and estriol are similar to those reported by others (1, 2). This can be more readily seen by expressing them in the form:

$$\frac{\text{cpm injected}}{\text{SA(metab.)} \times \text{t}}$$

where SA (metab.) = specific activity of the

TABLE 4. Specific activities (cpm/μg) of urinary metabolites of injected estradiol-6,7-^{3}H in subject SA calculated from successive crystallizations*

Metabolite†	Crystals 1	Crystals 2	Crystals 3	Crystals 4	ML 4	Crystals 5	ML 5
2-MeE$_1$	24	28	28	—	—	—	—
E$_1$	36	37	38	38	36	—	—
E$_2$	—	43	48	46	—	48	43
16α-OHE$_1$	24	25	27	24	26	—	—
16-KE$_2$	—	31	25	25	25	—	—
16-epiE$_3$	22	23	24	22	22	—	—
E$_3$	7	7	7	7	7	—	—

* Values are based on a dose of 5×10^6 cpm and are rounded off to the nearest whole number.
† See footnote 1 for abbreviations.

TABLE 5. Specific activities (cpm/μg) of urinary metabolites of injected estradiol-6,7-^{3}H in subject SH calculated from successive crystallizations*

Metabolite†	Crystals 1	Crystals 2	Crystals 3	Crystals 4	ML 4	Crystals 5	ML 5
2-MeE$_1$	—	46	50	—	—	—	—
E$_1$	—	58	55	62	—	58	59
E$_2$ (lost)	—	—	—	—	—	—	—
16α-OHE$_1$	35	39	38	39	35	—	—
16-KE$_2$	—	21	28	27	21	—	—
16-epiE$_3$	—	34	33	33	36	—	—
E$_3$	6	7	6	6	6	—	—

* Values are based on a dose of 5×10^6 cpm and are rounded off to the nearest whole number.
† See footnote 1 for abbreviations.

^{3}H-labeled metabolite in question and t = time over which urine was collected. This expression has been used to calculate the secretion rate of steroids, although it has been pointed out that in the complex situation associated with pregnancy such a calculation does not, in fact, provide a true measure of secretion rate (2). Bearing this in mind, the "secretion rates" (mg/24 hr) in the present study are: estrone 19–33, estradiol 16–27 and estriol 156–208.

The striking difference between the specific activities of urinary 16α-hydroxyestrone and estriol suggests that the former, in its endogenous form, is not principally derived from fetoplacental sources, at least in late pregnancy. Thus, if it were, one might expect the specific activities to be similar. That this is not so might conceivably relate to a very rapid turnover of 16α-hydroxyestrone within the fetoplacental unit. 16-Ketoestradiol is usually considered to be a metabolite of estriol in the human (20), while 16-epiestriol may be formed from 16β-hydroxyestrone or by reduction of 16-ketoestradiol (21). On the basis of our present results, it seems unlikely that 16-ketoestradiol and 16-epiestriol arise mainly from fetoplacental estriol in view of the similarity of their specific activities to that of 16α-hydroxyestrone. It should be pointed out that there is little evidence at present in favor of oxidative metabolism of estriol in pregnancy (22, 23).

The results obtained during the present work suggest that 16α-hydroxyestrone, 16-ketoestradiol and 16-epiestriol excreted in the urine in late pregnancy are largely the result of peripheral metabolism in the maternal tissues of secreted estrone and/or estradiol. However, some part of these may also be formed in a separate pool (perhaps as precursors and metabolites of fetoplacental estriol) to an extent which would explain the difference in specific activity of

TABLE 6. Specific activities (cpm/μg) of urinary metabolites of injected estradiol-6,7-^{3}H in subject IS calculated from successive crystallizations*

Metabolite†	Crystals 1	Crystals 2	Crystals 3	Crystals 4	ML 4	Crystals 5	ML 5
2-MeE$_1$	36	46	41	—	—	—	—
E$_1$	—	61	61	62	—	65	69
E$_2$	—	65	82	73	—	77	75
16α-OHE$_1$	41	44	42	43	—	44	43
16-KE$_2$	32	36	36	36	36	—	—
16-epiE$_3$	33	36	36	36	39	—	—
E$_3$	7	7	7	6	7	—	—

* Values are based on a dose of 5×10^6 cpm and are rounded off to the nearest whole number.
† See footnote 1 for abbreviations.

these three metabolites compared with those of urinary estrone and estradiol.

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SECTION C

METABOLISM/INTERCONVERSION OF FREE OESTROGENS IN VITRO AND IN VIVO

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CONVERSION OF 16-^{14}C-ESTRADIOL-17β TO ^{14}C-LABELLED ESTRIOL BY AVIAN LIVER SLICES

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The generally accepted pathway for estradiol - 17β metabolism as it involves the three classical estrogens is :

estradiol $\rightleftharpoons$ estrone $\longrightarrow$ estriol.

The conversion of estradiol to estriol is well established in vivo, this being an apparently irreversible reaction (Stimmel, 1947; Stealy and Stimmel, 1948). It is only recently that this transformation has been reported to occur in vitro, the tissues employed being human fetal liver (Engel et al, 1958) and rat liver (Hagopian and Levy, 1958). This conversion of estradiol is apparently of more than academic importance since it has been shown to proceed to a greater extent than normal in human males with prostatic cancer (May and Stimmel, 1948) and myocardial infarction (Bauld et al, 1957).

Certain evidence has accumulated in favour of the existence of an additional biosynthetic pathway for estriol involving neither estrone nor estradiol (Brown, 1957; Ryan, 1958). Besides these observations, interest in estriol has arisen from its apparent non-occurrence in a number of mammalian species. This is of particular interest in the rabbit which does not exhibit spontaneous atherosclerosis.

In view of the finding of estriol in avian excreta (Hurst, 1957) and also in ovarian extracts from the laying hen (Layne and Common, 1958) it seemed that in vitro experiments on hepatic tissue from such a source might yield significant results.

Experiments and Results

Slices of liver (500 mg.) obtained from a laying hen were incubated with 40 μg. of 16-^{14}C-estradiol-17β (2.7 μc./mg.) in 5 ml. of Krebs-Ringer-Phosphate solution, pH 7.4, for 2 hours at 37° with shaking. After incubation 1 mg. of pure unlabelled estriol was added as carrier. Following the preparation of a lipid extract a 4-transfer distribution in C_6H_6/H_2O was performed with single withdrawal, and subsequent saponification of the estriol-containing aqueous phase. Part of this material was subjected to paper chromatography in the system C_6H_6:C_6H_{14}(1:1)/CH_3OH:H_2O(7:3) followed by radioautography. This showed all of the radioactivity to be associated with a spot of the same chromatographic mobility as pure estriol. A second portion of the aqueous extract was subjected to a 24-transfer countercurrent distribution in $CHCl_3$:CCl_4(4:1)/CH_3OH:H_2O(7:3) yielding a curve for radioactivity which corresponded in position to that of the carrier estriol as measured by the Kober reaction (Fig. 1). The estriol

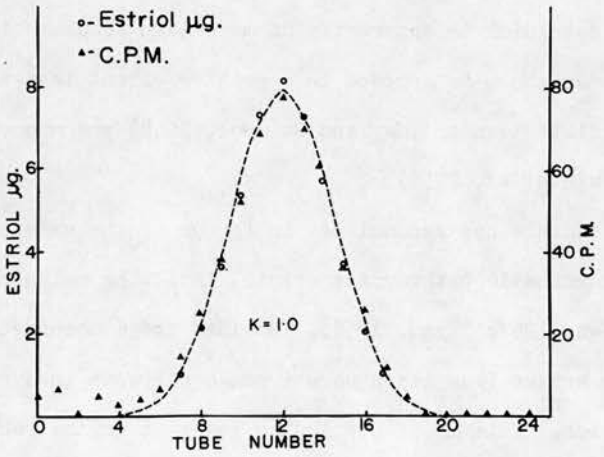

Fig. 1. 24-Transfer countercurrent distribution in 70% $CH_3OH/CHCl_3$:CCl_4 (4:1) of carrier estriol (open circles) and the radioactive product obtained after incubating 16-^{14} C-estradiol - 17β with avian liver (triangles). The broken line represents the theoretical distribution curve.

had a specific activity of 9,800 counts/min./mg. at this stage. The remainder of the material was chromatographed on paper in $CHCl_3/HCONH_2$ against pure estriol as standard. Elution of the estriol-containing zone

was followed by chromatography on a celite partition column (Bauld, 1956) in $ClCH_2CH_2Cl/CH_3OH:H_2O$. The resulting estriol had a specific activity of 10,000 counts/min./mg. Part of this was methylated to yield the methyl ether derivative which was subsequently partitioned in C_6H_6/H_2O. The organic extract, when chromatographed on alumina (Brown, 1955) yielded estriol methyl ether of specific activity 9,800 counts/min./mg.

Comment

The data outlined above constitutes good evidence for the conversion of estradiol - 17β to estriol by avian liver, thus indicating that some, at least, of the estriol associated with avian metabolism probably arises by this route. The extent of the conversion found here was approximately 6%.

One of us (J. E. M.) is the Lawrence de Coteau Research Fellow, Department of Metabolism, The Montreal General Hospital. This work was also supported in part by National Research Council of Canada Grant No. MT 532, and by a grant from Parke, Davis and Co.

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Interconversion of 16-Epioestriol and Oestriol by Avian Liver Tissue *in vitro*

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1. [16-^{14}C]16-Epioestriol and [6,7-$^{3}H_2$]oestriol were incubated both simultaneously and separately at 40° with hen liver homogenates obtained from birds in the laying stage. 2. Purification and identification of products was made by ether extraction, Girard separation, Celite partition and paper chromatography and crystallization to constant specific activity both with and without derivative formation. Percentage conversions of the radioactive 16-epioestriol and oestriol were calculated from these specific activities. 3. Oestriol yielded 16-epioestriol as early as 5min. after exposure of steroid to the tissue. No production of oestriol from 16-epioestriol was detectable in this time. 4. Considerably more 16-epioestriol was formed from oestriol than oestriol by the reverse reaction after 90min. of incubation. 5. 16-Oxo-oestradiol-17β appeared in all cases to be the major identified intermediate in the interconversion of 16-epioestriol and oestriol.

The endogenous metabolic pattern of steroidal hormones of the more common test animals has not been well defined. One species, however, about which considerable information has been made available in recent years is the fowl. It is remarkable that the laying hen possesses an oestrogen pattern which qualitatively bears a very strong resemblance to that of the human. Thus Ainsworth, Carter & Common (1962), on intravenous administration of [16-^{14}C]oestrone (3-hydroxy[16-^{14}C]-oestra-1,3,5-trien-17-one) to a laying hen recognized, chromatographically, radioactive oestradiol-17β (oestra-1,3,5-triene-3,17β-diol), oestriol (oestra-1,3,5-triene-3,16α,17β-triol) and oestrone in the urine. Other radioactive metabolites detected were 16-epioestriol (oestra-1,3,5-triene-3,16β,17β-triol), 17-epioestriol (oestra-1,3,5-triene-3,16α,17α-triol), 16-oxo-oestradiol-17β (3,17β-dihydroxyoestra-1,3,5-trien-16-one) and 16-oxo-oestrone (3-hydroxyoestra-1,3,5-triene-16,17-dione). Previously MacRae, Dale & Common (1960) had demonstrated the conversion of [16-^{14}C]-oestriol into radioactive 16-oxo-oestradiol-17β and 16-epioestriol in laying hens. No additional radioactive conversion product was detected in the urine. Although the above compounds were identified from chromatographic evidence, oestrone (Ainsworth & Common, 1962), oestradiol-17β (MacRae, Zaharia & Common, 1959) and 16-epioestriol (Hertelendy & Common, 1964) have also been fully characterized in crystalline form from hen urine or faeces or both. No oestriol has, however, been isolated.

Intravenous injection of labelled oestradiol-17β into the laying bird resulted in a radioactive oestriol/16-epioestriol ratio in excreta which was considerably lower than that found in human urine, where oestriol is a major metabolite (MacRae & Common, 1960; Marrian & Bauld, 1955; Hobkirk, Nilsen & Purre, 1966). After intravenous administration of 16-oxo[16-^{14}C]oestradiol-17β to the non-laying fowl, Ainsworth & Common (1963) identified only radioactive 16-oxo-oestradiol-17β and 16-epioestriol in the urine. This again suggested the equilibrium conditions in the hen to be such that 16-epioestriol is formed at the expense of oestriol.

On the other hand, Mitchell & Hobkirk (1959) demonstrated a significant conversion of radioactive oestradiol-17β into labelled oestriol by hen liver slices *in vitro*. Recently Ozon & Breuer (1965), by incubating 16-oxo-oestradiol-17β with liver slices from chicks of 10–20 days of age and immature pullets, have shown both 16-epioestriol and oestriol to be formed in the ratio 3:2 in favour of the former metabolite. They also demonstrated oestriol to be an incubation product of 16α-hydroxyoestrone (3,16α-dihydroxyoestra-1,3,5-trien-17-one) and of oestradiol-17β.

To obtain a more complete understanding of the interconversion of oestriol and 16-epioestriol *in vitro*, these two compounds, in radioactive form, have been incubated with liver preparations from

the laying hen and their metabolites investigated as described below.

EXPERIMENTAL

Materials

NAD, NADP and glucose 6-phosphate were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. ATP was obtained as the disodium salt from Sigma Chemical Co., St Louis, Mo., U.S.A. 16-Oxo-oestradiol-17β was acquired from Steraloids Inc., Pawling, N.Y., U.S.A. 16-Epioestriol was purchased from Organon Inc., W. Orange, N.J., U.S.A., and oestriol was a gift from Parke, Davis and Co., Ann Arbor, Mich., U.S.A. Dr A. E. Kellie of the Courtauld Institute of Biochemistry, The Middlesex Hospital, London, W. 1, donated the 16α-hydroxyoestrone.

[16-^{14}C]16-Epioestriol was prepared by chemical reduction of 16-oxo[16-^{14}C]oestradiol-17β of specific activity 0·04 μc/μg. as described by Hobkirk (1963). Successive crystallizations with authentic carrier steroid of a sample of the material eluted from a Celite partition column showed it to be 99% [16-^{14}C]16-epioestriol. It was used without further purification. The 16-oxo[16-^{14}C]oestradiol-17β was donated by Dr M. Levitz, New York University, New York, N.Y., U.S.A. [6,7-$^{3}H_2$]Oestriol of specific activity 52 μc/μg., purchased from New England Nuclear Corp., Boston, Mass., U.S.A., was purified by column partition chromatography and shown to be at least 98% pure by crystallization with pure unlabelled oestriol.

All other reagents were of suitable chemical grade and were purified where necessary by published techniques (Givner, Bauld & Vagi, 1960). Final distillation of organic solvents was done in all-glass apparatus and these solvents were stored in brown glass bottles.

Celite 535 (Johns–Manville Corp. New York, N.Y., U.S.A.) was purified, prepared and tested as described by Bauld & Greenway (1957).

Methods

Assay of radioactivity. All measurement of radioactivity was performed by liquid-scintillation spectrometry in a Nuclear–Chicago Corp. no. 6725 dual-channel instrument operating at an efficiency of 66% and 33% for ^{14}C and ^{3}H respectively. In double-label counting less than 1% of ^{3}H appeared in the ^{14}C channel while approx. 15% of ^{14}C appeared in the ^{3}H channel. The radioactivity for all samples was determined to within 5% probable error. The material to be counted was dried in glass counting vials and dissolved in 0·2ml. of methanol before dilution with 10ml. of toluene containing 0·3% of 2,5-diphenyloxazole and 0·01% of 1,4-bis-(5-phenyloxazol-2-yl)benzene. Calculation of ^{14}C and ^{3}H from double-labelled samples was done according to the method of Okita, Kabara, Richardson & LeRoy (1957).

Preparation of homogenate. This was carried out as partially described by Mitchell & Hobkirk (1959) and Lucis (1965). Livers from White Rock hens, in the laying period at the time of decapitation, were excised, rinsed free of blood, weighed and immediately used. Krebs–Ringer phosphate solution, pH 7·4 (Krebs & Henseleit, 1932), modified to contain K_2HPO_4 (0·1 M) rather than Na_2HPO_4 (0·1 M) and fortified with 0·04 M-nicotinamide, was used in the proportion of 1ml./100mg. wet wt. of whole liver tissue. The tissue was homogenized for 30sec. in a VirTis 45 homogenizer.

Simultaneous incubation of ^{14}C- and ^{3}H-labelled substrates. To a series of 20ml. liquid-scintillation spectrometer vials (Nuclear–Chicago Corp., Toronto, Canada) were added $9·1 \times 10^5$ counts/min. of [6,7-$^{3}H_2$]oestriol and $9·9 \times 10^5$ counts/min. of [16-^{14}C]16-epioestriol in methanol. In each case an appropriate amount of unlabelled pure standard steroid, also dissolved in methanol, was added to give a final weight of 50μg. of each substrate. Propylene glycol (0·1ml.) was added to each vial and the methanol completely evaporated in a gentle stream of air. In addition to the substrates, cofactors were added as follows: NAD (2·3μmoles), NADP (2μmoles), glucose 6-phosphate (15μmoles), ATP (10μmoles) and $MgCl_2$ (279μmoles).

To each of the vials was added 5ml. of the above liver homogenate and incubation was carried out at 40° in an atmosphere of O_2+CO_2 (95:5) with regular mechanical agitation in a Dubnoff metabolic incubator. Times of incubation varied from 5 to 90min. Blanks were identical except for boiling of the homogenate for 20min. before addition to the incubation mixture.

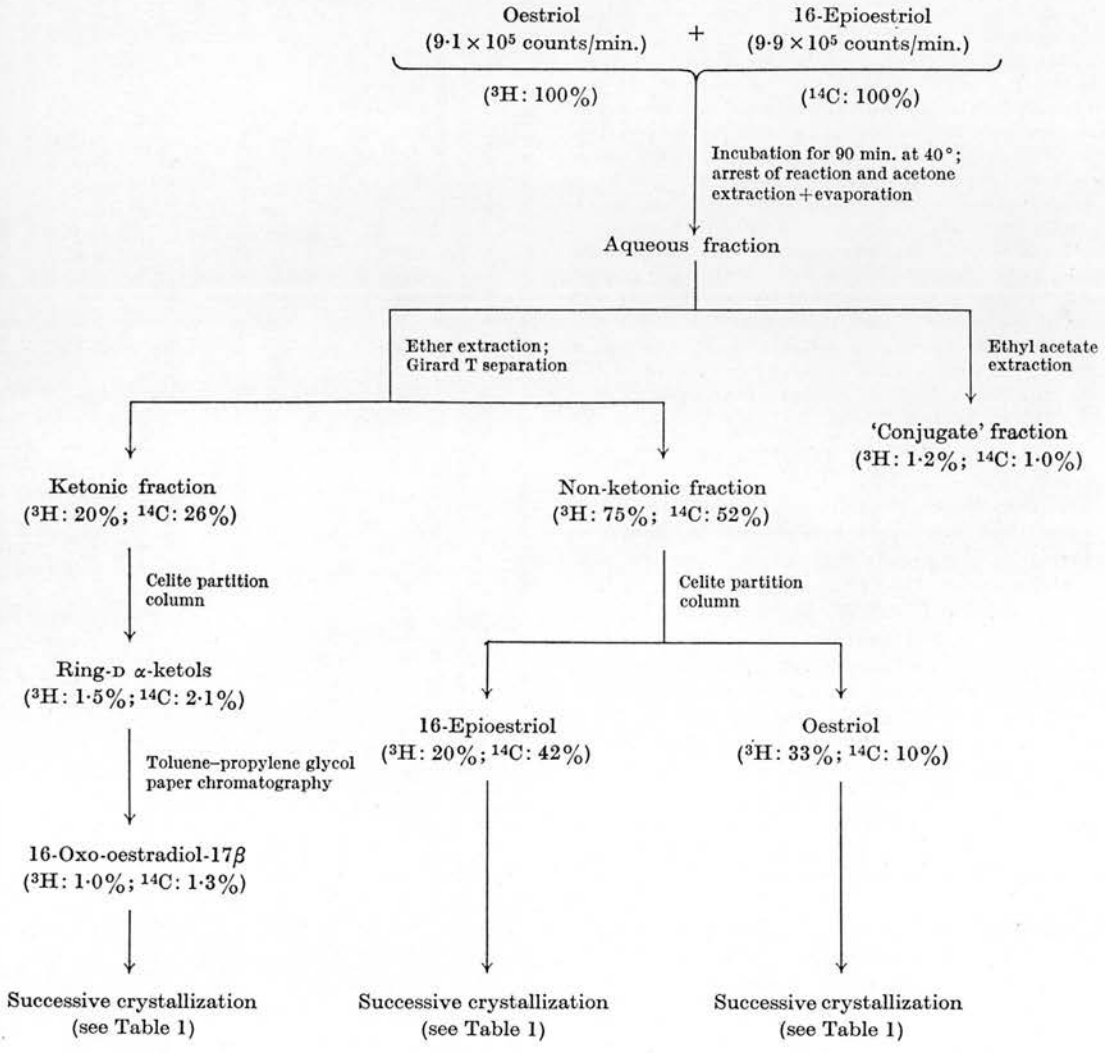
Extraction of free radioactive material from the incubation mixture. After incubation, 100μg. each of unlabelled oestriol, 16-epioestriol, 16-oxo-oestradiol-17β and 16α-hydroxyoestrone were added to the incubation medium as carriers. Simultaneously the protein of the mixture was precipitated by the addition of 50ml. of acetone. The mixture was kept overnight at room temperature (24°) to complete the extraction. The acetone was removed by filtration (Whatman no. 31 filter paper) and the tissue residue rinsed with additional acetone (four 40ml. portions). The washings and acetone extract were combined with 50ml. of distilled water and the acetone was evaporated under reduced pressure. The aqueous residue was then extracted with peroxide-free ether (four 60ml. portions). The ether was evaporated off and the extracts were further dried by placing over anhydrous $CaCl_2$ in a vacuum desiccator overnight. The aqueous portions of each sample were stored for later extraction of conjugated material.

Purification and characterization of the radioactive incubation products. The dried ether extracts were subjected to a modified Girard separation for 18hr. at room temperature (24°) as described by Givner *et al.* (1960). The ketonic and non-ketonic fractions, after ether extraction, were evaporated to dryness, dissolved in methanol and a sample (one-hundredth) from each was reserved for assay of radioactivity. The remaining material was chromatographed on Celite partition columns according to the method of Givner *et al.* (1960). This resulted in the separation of fractions containing oestriol, 16-epioestriol and ring-D α-ketolic oestrogens. [The last fraction would consist of any 16α-hydroxyoestrone, 16β-hydroxyoestrone (3,16β-dihydroxyoestra-1,3,5-trien-17-one) and 16-oxo-oestradiol-17β formed during incubation.] Radioactive eluates corresponding to each of these chromatographic fractions were evaporated to dryness and redissolved in methanol (3·0ml.). One-tenth of each was retained for counting. A larger sample (two-thirds) was taken for further purification and identification.

Successive crystallization of radioactive 16-epioestriol and oestriol. The eluates obtained from the partition columns were submitted to a final step for the achievement of radiochemical purity. Pure crystalline unlabelled carrier of the

appropriate type was added to all 16-epioestriol and oestriol fractions and the mixtures were acetylated in pyridine and acetic anhydride overnight. The samples were successively crystallized several times from methanol–water. The specific activities of the crystals (approx. 1 mg. accurately weighed was counted in each case) became constant and equal to that of the mother liquor on the second crystallization.

Characterization of the radioactive ring-D α-ketolic fraction. The ring-D α-ketols were examined by descending paper chromatography in a toluene–propylene glycol solvent system at 24° (Hobkirk *et al.* 1966). Whatman no. 42 paper was impregnated with propylene glycol–methanol (1:1, v/v), blotted to remove excess of solvent and dried in air sufficiently long to remove methanol before chromatography. The ring-D α-ketolic fraction from the Celite column was applied to the starting line with small volumes of methanol along with pure 16α-hydroxyoestrone (40 μg.) and 16-oxo-oestradiol-17β (40 μg.) as standards. The paper was developed for 4 days (overflow collected) in a jar previously saturated with toluene for 24 hr. Radioactive areas corresponding to standards were eluted with methanol overnight. The eluates were filtered through sintered glass, evaporated and redissolved in methanol (4·0 ml.). One-tenth was retained for counting. The remaining nine-tenths of the radioactive fractions eluted from the paper was transferred to centrifuge tubes and crystallized with pure unlabelled carrier from acetonitrile as described by Levitz,



Scheme 1. Typical distribution of radioactivity during different stages of extraction and purification of incubation products. An example from a [16-^{14}C]16-epioestriol + [6,7-$^{3}H_2$]oestriol experiment after 90 min. incubation is shown (see the Methods section). Figures are given as percentages of incubated radioactivity.

Spitzer & Twombly (1956). The crystals were of constant specific activity and equal to that of the mother liquor on the second recrystallization. From the specific activities thus obtained and knowing the weights of carrier used, percentage conversions of the [6,7-$^3H_2$]oestriol and [16-^{14}C]-16-epioestriol were calculated.

Extraction of possible conjugates. Although conjugation in itself was not of major interest, the residual aqueous fraction after ether extraction of free radioactivity was treated as follows. The water fraction was gently heated to remove any ether, cooled, 3M-NaCl added and the pH adjusted to 2. After extraction with 3vol. of ethyl acetate (Burstein & Lieberman, 1958) the extracts were dried over Na_2SO_4 and evaporated *in vacuo*. The dry residue was dissolved in methanol (5·0ml.) and a sample removed for assay of radioactivity. The degree of 'conjugation' in this tissue system was so low (0·5–2·0% of incubated substrate) that extraction of this fraction was dispensed with in later experiments.

Additional incubations. Confirmation of results obtained from the experiment outlined above was sought by carrying out further incubations with only a single substrate in any one experiment and additional times of incubation. Two series of incubations of this type were done. In the first of these $1{\cdot}0\times10^6$ counts/min. of [6,7-$^3H_2$]oestriol plus 50μg. of unlabelled oestriol were incubated for 1, 5, 30 and 90min. Secondly, $8{\cdot}6\times10^5$ counts/min. of [16-^{14}C]16-epioestriol plus 50μg. of unlabelled 16-epioestriol were incubated for 90min. only. Suitable blanks were carried out in each case. All other experimental details were identical with those of the double-labelled study described above. The three series of experiments outlined were performed on different occasions with livers excised from three individual birds. In all cases duplicate incubations were performed at each different incubation time.

RESULTS

The distribution of radioactivity obtained in a typical double-labelled experiment is shown in Scheme 1. Significant amounts of activity were associated with the 16-epioestriol, oestriol and ring-D α-ketolic fractions. As shown in Table 1, virtually all of the radioactivity in the two triol fractions remained associated with the carriers during crystallization. However, on subfractionation of the ring-D α-ketolic fraction, in no case was radioactivity found in the position of 16α-hydroxyoestrone or 16β-hydroxyoestrone. In all experiments, 16-oxo-oestradiol-17β eluted from chromatograms and crystallized as typified by Table 1 was the main metabolite. It accounted for 61–79% of the total ^3H of the ring-D α-ketolic fraction after [6,7-$^3H_2$]oestriol incubation at various times and 65–100% of the ketolic ^{14}C when [16-^{14}C]16-epioestriol was the substrate. The rest of the radioactivity remained on the starting line of the chromatograms and was not further investigated. Table 2 indicates that oestriol may be metabolized to 16-epioestriol very rapidly. In the first 5min. of incubation (Expt. A) 15·9–17·5% of the oestriol was transformed into the latter metabolite. After

Table 1. *Successive crystallization of radioactive incubation products of hen liver homogenate*

A typical purification of radioactive oestriol triacetate, 16-epioestriol triacetate and 16-oxo-oestradiol-17β is shown. Recrystallization of the acetates was done from methanol–water and the 16-oxo-oestradiol-17β was crystallized from acetonitrile. The example was from a [16-^{14}C]16-epioestriol+[6,7-$^3H_2$]-oestriol incubation at 90min. Experimental details are given in the text. Weights and specific activities are in terms of the free steroids.

	Oestriol			16-Epioestriol			16-Oxo-oestradiol-17β			
		Sp. activity (counts/min./mg.)			Sp. activity (counts/min./mg.)			Sp. activity (counts/min./mg.)	
Purification step	Wt. (mg.)	^{14}C	^3H	Purification step	Wt. (mg.)	^{14}C	^3H	Purification step	Wt. (mg.)	^{14}C	^3H
Celite-column pool +20mg. of carrier	20·0	4960	15140	Celite-column pool +20mg. of carrier	20·0	20350	9200	Paper-chromatographic pool +20mg. of carrier	20·0	670	432
Acetylation with acetic anhydride and pyridine				Acetylation with acetic anhydride and pyridine							
Crystals 1	13·3	4210	14480	Crystals 1	16·4	20060	9080	Crystals 1	11·3	561	372
Mother liquor 1	5·7	6260	15610	Mother liquor 1	3·1	20000	8880	Mother liquor 1	8·3	698	456
Crystals 2	9·2	4020	13760	Crystals 2	14·8	20370	8870	Crystals 2	5·7	586	373
Mother liquor 2	3·9	4090	13900	Mother liquor 2	1·6	20180	8820	Mother liquor 2	5·7	591	390
Crystals 3	5·5	4020	13800	Crystals 3	12·8	20390	9050				

30 and 90min. incubation periods the amount of 16-epioestriol had decreased but still remained significantly high. This decrease was not reproducible on repetition of the study with liver tissue obtained from another bird. Nevertheless, 5·2–6·2% of 16-epioestriol was again formed after 5min. of exposure of oestriol to the tissue (Table 2, Expt. B). After 90min. of incubation 19·5% of oestriol had been metabolized to the epimer. In all cases a decrease in the percentage recovery of unchanged oestriol accompanied increased 16-epioestriol production. Material identified as 16-oxo-oestradiol-17β derived from oestriol also increased with enhanced 16-epioestriol formation.

When 16-epioestriol was the substrate (Table 3), duplicate 5min. incubations yielded no detectable oestriol. Considerable conversion (8·1–8·6%) occurred at 90min., however. A repeat of this experiment on another occasion with a new liver preparation again yielded oestriol (Table 3, Expt. C), but less in this case (2·7–3·1% at 90min.). It is noteworthy that 16-oxo-oestradiol-17β appeared

Table 2. *Percentage conversion of* [6,7-$^3H_2$]*oestriol into labelled* 16-*epioestriol and* 16-*oxo-oestradiol*-17β *at various times of incubation*

Incubation, extraction and purification of products were performed as described in the text. The percentage of substrate converted and unchanged was calculated from the product of the specific activity of crystals 2 (cf. Table 1) and the weight of carrier (20mg.) added. Expts. A and B designate separate incubations with livers from different laying hens; (1) and (2) represent duplicate incubations of the same preparation.

Expt. no.	Time of incubation (min.)	Conversion into 16-epioestriol (%)	Conversion into 16-oxo-oestradiol-17β (%)	Unchanged substrate (%)
A (1)	1	1·7	0·06	31·9
(2)	1	2·5	0·07	33·0
A (1)	5	17·5	0·10	17·7
(2)	5	15·9	0·09	18·1
A (1)	30	5·6	—	—
(2)	30	6·0	—	—
A (1)	90	8·1	1·2	9·4
(2)	90	8·3	1·4	9·8
A (1)	Blank	2·2	0·04	40·2
(2)	Blank	2·6	0·06	32·9
B (1)	5	6·2	0·14	56·6
(2)	5	5·2	0·12	57·0
B (1)	90	19·5	0·82	30·3
(2)	90	19·5	0·84	28·9
B (1)	Blank	1·7	0·01	52·8
(2)	Blank	2·2	0·02	53·2

Table 3. *Percentage conversion of* [16-^{14}C]16-*epioestriol into labelled oestriol and* 16-*oxo-oestradiol*-17β *at various times of incubation*

Incubation was performed for 5 and 90min. only. All other details are as given in Table 2. Expts. B and C designate separate incubations with livers from different laying hens; (1) and (2) represent duplicate incubations of the same preparation.

Expt. no.	Time of incubation (min.)	Conversion into oestriol (%)	Conversion into 16-oxo-oestradiol-17β (%)	Unchanged substrate (%)
B (1)	5	0	0·35	79·5
(2)	5	0	0·40	69·9
B (1)	90	8·1	1·2	41·2
(2)	90	8·6	1·4	40·1
B (1)	Blank	0	0·02	71·3
(2)	Blank	0	0·03	72·2
C (1)	90	2·7	—	39·4
(2)	90	3·1	9·3	43·0
C (1)	Blank	0	0·15	50·0
(2)	Blank	0	0·21	53·2

quantitatively related to increased product formation once more, with one exception. 16-Epioestriol incubation at 90min. with tissue from one bird resulted in 1·2–1·4% 16-oxo-oestradiol-17β formation. Tissue obtained from another bird, however, yielded 9·3% of the ketol under the same conditions. As product identification was of primary interest in this work such discrepancies between liver activites of different birds were not too disturbing.

It is noted that consistently higher amounts of 16-oxo-oestradiol-17β were recovered when 16-epioestriol was metabolized to oestriol than during the reverse reaction. Also, it appears that a larger fraction of the ring-D α-ketolic compounds was identifiable as 16-oxo-oestradiol-17β when 16-epioestriol was the substrate than on oestriol incubation. Oestriol appeared to yield a larger percentage of more polar unidentified material in this fraction.

DISCUSSION

The above findings in relation to the interconversion of oestriol and 16-epioestriol by liver homogenates from the laying hen supplement in part the studies *in vivo* of MacRae *et al.* (1960) and Ainsworth & Common (1963). As mentioned above, these authors showed 16-epioestriol to be the major triol excreted by the fowl whereas oestriol was either scarce or undetectable.

Considerably more 16-epioestriol was formed *in vitro* from oestriol than oestriol by the reverse reaction, at least under the experimental conditions employed. Further, the 16α-to-16β epimerizing systems appeared to be more rapid-acting, converting oestriol into 16-epioestriol almost immediately on contact of substrate and tissue. The 16β-to-16α epimerization did not appear nearly so active. This evidence *in vitro* appears to be in agreement with the results of Common and co-workers. These authors, however, did not detect any oestriol formation from 16-epioestriol *in vivo*. The present work has shown this pathway to exist *in vitro*. We have also shown that 16-oxo-oestradiol-17β appears in all cases to be the major identified intermediate in oestrogen triol interconversion, a fact that has partially been borne out by Ozon & Breuer (1965) on incubation of 16-oxo-oestradiol-17β by hen livers and isolation of both epimers.

It should be indicated that recovery of radioactivity in these experiments never reached 100%. Blank values in Tables 2 and 3 indicate cases where only 50% or less of the material incubated was extractable in unchanged form. Such a low recovery may have resulted from the cumulative effect of tissue binding, experimental loss at different stages of purification and other factors resulting in underestimation of the degree of metabolism.

In conclusion, it must be stated that up to the present time only a few tissues in a limited number of species have been shown to metabolize oestrogen triols to a significant degree. King (1960) indicated phenolic triol interconversion by rat kidney preparations. Hobkirk, Nilsen & Belenkie (1965) have demonstrated the dehydrogenation of oestriol to 16-oxo-oestradiol-17β by cat erythrocytes and considerable dehydrogenation of 16-epioestriol by rat erythrocytes. The latter has also been reported by Portius & Repke (1960). The results of the present work now show that homogenates prepared from the livers of laying hens are also capable of effecting such conversions.

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Reduction of 16-Ketoestradiol-17β 16-^{14}C by Mammalian Blood*

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Reduction of the ketonic group of 16-ketoestradiol-17β[1] by mammalian erythrocytes *in vitro* has been reported by two groups of investigators. Breuer (1), employing human red cells, showed the reduction product to be 16-epiestriol (16β reduction) in a yield of 0.8%. Portius and Repke (2) also found 16-epiestriol to be the transformation product when erythrocytes of human, rat, and bovine origin were used. A preliminary report from our laboratory (3) dealt with an apparent species difference in this reduction, the major product being estriol (16α reduction) in cat erythrocytes and, in agreement with the other investigators, 16-epiestriol in rat cells. This paper represents an extension of our preliminary work.

EXPERIMENTAL PROCEDURE

Materials—All chemical reagents and organic solvents were of good reagent grade and were purified where necessary by previously published procedures (4–6). 16-Ketoestradiol-17β 16-^{14}C was synthesized by Levitz and Spitzer (7) and was chromatographed on Celite partition columns and paper to yield material of at least 98% purity.

Whole blood (heparinized) was obtained from cat, cow, dog, human, pig, rat (Wistar), and sheep. In each case, females were chosen, but no consideration was given to gonadal function.

Methods—Whole blood, plasma, erythrocytes, or leukocyte suspensions were incubated in Erlenmeyer flasks with labeled substrate dissolved in 1 to 2 drops of propylene glycol for 30 minutes or 2 hours in a Dubnoff shaking incubator at 37°. The gas phase was 95% O_2-5% CO_2 or 100% O_2.

Where radioactivity was to be measured in plasma and erythrocytes separately following whole blood incubation, the separated plasma was extracted 3 times each with 1 volume of diethyl ether, and the washed red cells were hemolyzed with ether-ethanol (3:1). Where identification of metabolites was to be made, the incubation was terminated by adding 5 to 10 volumes of acetone-ethanol (1:1), and the mixture was allowed to stand overnight at 4°. The supernatant and washings of the precipitated residue were combined, the volatile solvent was removed under reduced pressure, and the residual aqueous phase, after dilution with water to 50 or 100 ml, was extracted with ether as before. These extracts were washed with M sodium bicarbonate, then with water, and dried over anhydrous sodium sulfate before being evaporated to dryness. The residue was partitioned once between 90% methanol and hexane (Experiment B). The 90% methanol fraction, or the total ether-extractable residue (Experiment A), was subjected to a modified Girard separation (6), and the nonketonic fraction was purified by alkaline treatment (8).

Estriol and 16-epiestriol fractions were separated out of the nonketonic material by Celite column partition (6). In Experiment A, these two fractions were each partitioned between benzene and water to exclude contamination of one compound by the other. For further identification, the estriol fractions were diluted with milligram amounts of carrier estriol followed by crystallization before and after acetylation or methylation (4). 16-Epiestriol fractions were diluted with smaller amounts of carrier and purified by crystallization, methylation, acetonide formation (9), and chromatography on a 15-cm Celite column in the solvent system ethylene dichloride-80% methanol. Weight of 16-epiestriol was determined by the Ittrich spectrophotometric procedure (10) with tetrachloroethane as solvent.

In all of the study except Experiment B, radioactivity of plated samples was measured in the Geiger region with a Nuclear-Chicago model D-47 gas flow counting tube with thin end window. Efficiency of counting for ^{14}C was 19%. Correction for self-absorption was made, where necessary, by the method of Engel *et al.* (11). In Experiment B, a Packard Tri-Carb model 314AX liquid scintillation spectrometer, operating at an efficiency of 70% for ^{14}C, was employed. The material to be counted was dissolved in 0.1 ml of methanol and diluted with 5 ml of toluene containing 0.3% of 2,5-triphenyloxazole and 0.01% of 1,4-bis-2′-(5′-phenyloxazolyl)benzene.

A sufficient number of counts were accumulated for each sample to ensure a counting error of less than 5%.

RESULTS

Distribution of Radioactivity in Blood—Table I shows the distribution of radioactivity following incubation of the substrate with whole blood from seven species. The larger part of this activity was always found in the plasma, most of it being ether-extractable. Only small amounts were to be found associated with the erythrocytes. The largest degree of substrate reduction occurred in blood from rat, cat, and sheep. Preliminary thin layer chromatography (12), followed by elution and radioactivity measurement of zones corresponding in mobility to those of estriol and 16-epiestriol, showed that, with the exception of cat blood, the main (or only) reduction product was 16-epiestriol. Of the more easily accessible blood types, those of the cat and the rat represented the greatest extremes in the ratio 16α:16β reduction products.

Site of Reduction—Incubation of the labeled substrate with plasma, leukocyte suspension, and erythrocyte suspension in blood from the rat and the cat showed the reductive activity to

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[1] The trivial names used in the text and their systematic counterparts are: 16-ketoestradiol-17β, 3,17β-dihydroxyestra-1,3,(10)-trien-16-one; estriol, 3,16α,17β-trihydroxy-estra-1,3,5(10)-triene; 16-epiestriol, 3,16β,17β-trihydroxy-estra-1,3,5(10)-triene.

TABLE I

Distribution of radioactivity in blood after incubation with 16-ketoestradiol-17β 16-^{14}C

Substrate, 10^5 c.p.m. and 10 μg, was incubated with 25 ml of whole blood for 2 hours at 37°.

Species	Blood fraction							
	Plasma ether extract			Plasma residue		Erythrocytes		
	c.p.m. × 10^{-4}	*% original*	*NK:K**	*c.p.m.* × 10^{-4}	*% original*	*c.p.m.* × 10^{-4}	*% original*	*NK:K*
Cat	4.64	46.4	3.0	1.09	10.9	0.52	5.2	10.4
Cow	5.86	58.6	0.07	2.15	21.5	0.10	1.0	0.3
Dog	4.71	47.1	0.06	2.72	27.2	0.56	5.6	0.05
Human	5.00	50.0	0.26	2.25	22.5	0.33	3.3	0.16
Pig	5.64	56.4	0.26	1.64	16.4	0.19	1.9	0.56
Rat	7.39	73.9	5.5	1.63	16.3	0.13	1.3	0.84
Sheep	6.28	62.8	7.5	1.69	16.9	0.16	1.6	5.7
Average		56.5			18.8		2.8 (total = 78%)	

* NK:K = ratio of nonketonic to ketonic counts.

TABLE II

Distribution of radioactivity following incubation of 16-ketoestradiol-17β 16-^{14}C with whole blood

Experiment	Species	Blood volume	Incubated substrate		Recovered radioactivity				
					Ether-soluble		Ketonic	Nonketonic	
		ml	*μg*	*c.p.m.* × 10^{-4}	*c.p.m.* × 10^{-4}	*% original*	*c.p.m.* × 10^{-4}	*c.p.m.* × 10^{-4}	*% original*
Saline blank			3.6	5.84	5.80	99.3	4.20	0.140	2.4
A	Cat	35	3.6	5.84	5.19	88.9	1.50	2.655	45.5
A	Rat	20	3.6	5.84	4.95	84.8	0.675	3.815	65.4
B	Cat	15	0.3	1.31	1.025	78.0	0.111	0.795	60.7
B	Rat	15	0.3	1.31	1.025	78.0	0.048	0.965	73.6

TABLE III

Purification of estriol (cat) and 16-epiestriol (rat) in Experiment A

All crystallizations were done from methanol. Specific activities of the methyl ethers were calculated in terms of weight of free estrogen.

Estriol (cat)		16-Epiestriol (rat)	
Purification step	Specific activity	Purification step	Specific activity
	c.p.m./mg		*c.p.m./μg*
Radioactivity from benzene-water + 15.7 mg of carrier	478	Radioactivity from benzene-water + 840 μg of carrier	15.5
Crystals 1	494	Crystals 1	14.4
Mother liquor 1	482	Mother liquor 1	14.8
Crystals 2	495	Methyl ether	15.1
Mother liquor 2	465		
Methylation + extraction (4)			
Crystals 1	432		
Crystals 2	446		

be associated with the latter fraction. Packed red cells, 2.5 to 3.0 ml in an equal volume of 0.9% NaCl solution, reduced the substrate to the extent of 25% and 50% in cat and rat, respectively. The degree of reduction was found to be approximately as great after 30 minutes of incubation as after 2 hours.

Experiments A and B

The amounts of blood and substrate incubated, and the distribution of radioactivity following the initial fractionation, are shown in Table II. The apparent reduction was considerable in both experiments.

Purification of Products

Experiment A—After benzene-water partition of the extract from cat blood, 8500 c.p.m. were found in the aqueous phase. Of this activity, 7500 c.p.m. were diluted with carrier estriol and purified as shown in Table III. With the final specific activity taken to be 440 c.p.m. per mg, the apparent conversion to estriol following benzene-water treatment, and neglecting losses occurring prior to this step, was calculated to be 13%. The radioactivity in the 16-epiestriol fraction from the same experiment amounted to 1700 c.p.m., 1540 c.p.m. of which were diluted with 1.33 mg of carrier 16-epiestriol. Following two crystallizations from methanol, the specific activity fell from an initial value of 1160 c.p.m. per mg to 45 c.p.m. per mg. Thus, if any conversion to this compound occurred, it was extremely slight.

In the rat blood experiment, 26,000 c.p.m. were associated with the 16-epiestriol fraction following benzene-water partition. Dilution of 13,000 c.p.m. with carrier 16-epiestriol was followed by purification as shown in Table III. If the specific activity of the purified material is taken to be 14.5 c.p.m. per μg, it can be calculated that at the stage of benzene-water partition the conversion was 42%. The radioactivity associated with the estriol

fraction amounted to only 1,000 c.p.m. This, and similar fractions from separate experiments were combined giving 2,185 c.p.m. and diluted with 5.99 mg of carrier estriol. After three crystallizations from methanol, the specific activity of the crystals fell from 365 c.p.m. per mg to 55 c.p.m. per mg. This excluded the possibility of considerable substrate conversion to estriol.

Experiment B—Fig. 1 shows the pattern of radioactivity and

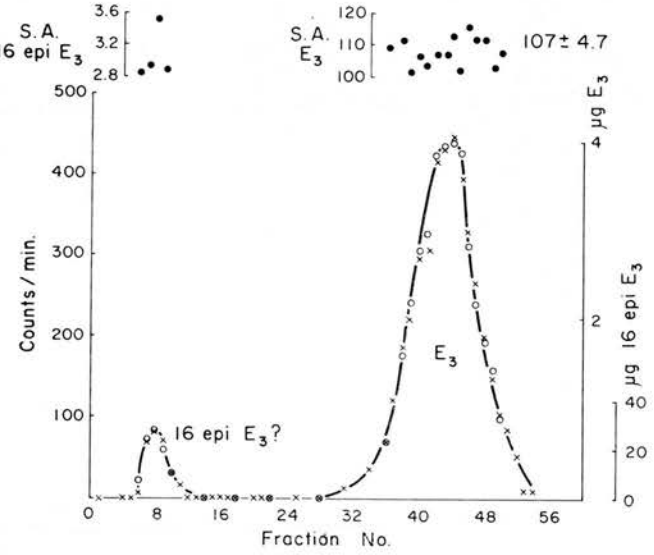

FIG. 1. Celite column chromatography of the nonketonic fraction from cat blood; Experiment B. The stationary phase was 70% methanol and the mobile phase benzene. ×——×, counts per minute; ○——○, weight. *S.A.*, specific activity; *16-epiE*$_3$, *16*-epiestriol; *E*$_3$, estriol. Fractions of 3 ml each were collected.

TABLE IV

Purification of estriol (cat) and 16-epiestriol (rat) in Experiment B

Crystallization of free estrogens was done from methanol and of acetates from methanol-water. Specific activities of the acetates were calculated in terms of the free estrogen.

Estriol (cat)		16-Epiestriol (rat)	
Purification step	Specific activity	Purification step	Specific activity
	c.p.m./mg		*c.p.m./μg*
Celite column pool + 15.22 mg of carrier	144	Celite column pool + 116 μg of carrier; added before Girard	55.6
Crystals	110	Acetonidation, re-extraction, hydrolysis (9)	51.4
Mother liquor	136	Celite column, ethylene dichloride-80% methanol	54.7*
Acetylation with acetic anhydride and pyridine			
Crystals 1	118		
Mother liquor 1	116		
Crystals 2	118		
Mother liquor 2	119		

* Average of 4 tubes of fraction collection; range = 52 to 59 c.p.m. per μg.

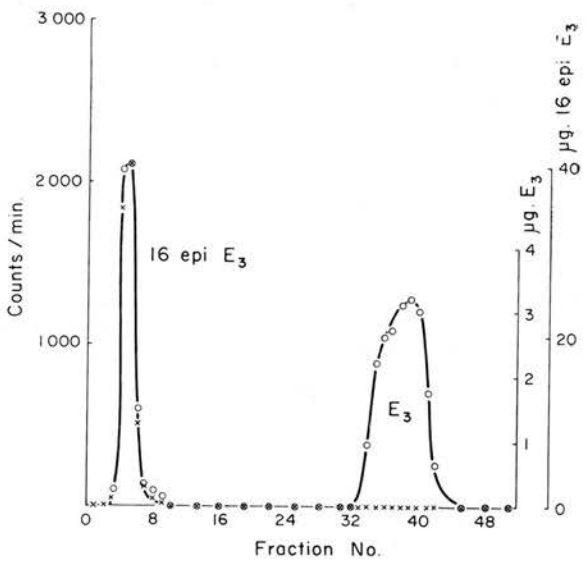

FIG. 2. Celite column chromatography of the nonketonic fraction from rat blood; Experiment B. The stationary phase was 70% methanol and the mobile phase benzene. ×——×, counts per minute; ○——○, weight. *16-epiE*$_3$, 16-epiestriol; *E*$_3$, estriol. Fractions of 4 ml each were collected.

weight following the chromatographic separation of 16-epiestriol (116 μg of carrier added prior to Girard separation) and estriol (50 μg of carrier added prior to Girard separation) from the nonketonic fraction of the cat blood experiment. Eluates containing the estriol peak were pooled (2190 c.p.m.) and diluted with carrier prior to the purification shown in Table IV. Some purification was achieved by the first crystallization. Calculation on the basis of the final specific activity showed an apparent conversion to estriol of 35% at the stage of ether extraction following incubation. A pool from the 16-epiestriol peak had a specific activity of 4.2 c.p.m. per μg, which on acetonide formation, re-extraction, and hydrolysis remained substantially unchanged at a value of 4.6 c.p.m. per μg. No further purification was attempted because of lack of radioactivity.

The pattern of carrier weight and radioactivity following partition column chromatography of the rat blood nonketonic fraction is shown in Fig. 2. The addition of carriers was the same as for the cat blood. The pooled 16-epiestriol peak was purified as shown in Table IV. The final specific activity was equivalent to a conversion to 16-epiestriol of 55%. Pooling of the estriol-containing fractions showed no trace of radioactivity.

DISCUSSION

The above findings in regard to the reduction of 16-ketoestradiol-17β to 16-epiestriol by rat blood are in agreement with those of Portius and Repke (2). However, these investigators do not seem to have revealed the extent of this reduction nor did they provide detailed information on identification of the product. It is quite evident that the extent of reduction can be considerable and that in cat blood the 16α isomer, estriol, is formed. Formation of the opposite isomer in each species either does not occur or does so only to a slight extent. The radioactivity that appeared in the cat 16-epiestriol fraction and the rat estriol fraction in Experiment A may have resulted through contamination by the

main metabolite due to interference by blood constituents not removed prior to column partition. Thus, the incubated volumes were rather large in this experiment, and no preliminary hexane-90% methanol partition was performed.

The question of the possible formation of additional metabolites is of interest. In Experiment B, although 61% and 74% reduction apparently occurred in cat and rat blood, respectively, the actual formation of estriol and 16-epiestriol was only 36% and 55% as estimated by isotope dilution. The difference could perhaps be accounted for by metabolites of a more water-soluble nature such as may have been responsible for that radioactivity found to remain in the plasma of seven species following ether extraction.

SUMMARY

Of seven mammalian species studied with respect to the activity of their blood to reduce 16-ketoestradiol-17β 16-^{14}C, the cat and the rat were found to be extremely active. The reduction product in the cat was estriol (16α isomer) and in the rat 16-epiestriol (16β isomer). Formation of the opposite C-16 isomer in each case was minimal if it occurred at all. Proof of identification of the products has been described.

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REVERSIBLE DEHYDROGENATION OF ESTROGEN TRIOLS BY SOLUBLE PREPARATIONS OF RAT AND CAT ERYTHROCYTES[1]

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It has been reported in previous publications from this laboratory (1, 2) that intact erythrocytes of rat and cat origin can reduce 16-KE_2-16-C^{14} to 16-epiestriol[2] (16β reduction) and estriol (16α reduction), respectively, as major conversion products. In further unpublished experiments it was not possible to demonstrate the reversal of these reactions in the presence of whole cells. Moreover, the use of unfractionated red cell hemolyzates proved unsuitable, probably as a result of binding of substrates or reaction products to protein, since the recovery of extractable radioactivity was markedly low after incubation. Soluble preparations of erythrocytes, which are active in catalyzing dehydrogenation of the triols and for which the cofactor requirement has been ascertained, have now been obtained.

Materials and Methods

All chemical reagents and organic solvents were of good reagent grade and were purified, where necessary, by published methods (3–5). NADP, NADPH, NAD, and NADH were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, or Sigma Chem. Co., St. Louis, Missouri. 16-KE_2-16-C^{14} (44 μcuries/mg) and estriol (22 μcuries/mg) were purified by column, paper, or thin-layer chromatography to yield material of at least 98% purity. 16-Epiestriol-16-C^{14} was prepared by chemical reduction of 16-KE_2-16-C^{14} as described elsewhere (6). Estrone-16-C^{14} (Radiochemical Centre, Amersham, England; 45 μcuries/mg) and dehydroisoandrosterone (Merck, Sharpe and Dohme Ltd.; 140 μcuries/mg) were sufficiently pure for direct use, as were all unlabelled steroids used. Whole blood (heparinized) was obtained from mature female cats and white Wistar female rats.

Fractionated rat cell hemolyzates were prepared at 4 °C by treating packed erythrocytes with H_2O. To the mixture was added 0.5 volume of aqueous $(NH_4)_2SO_4$ saturated at 4 °C to give 33% saturation. The precipitated material was discarded and the supernatant adjusted to 66% salt saturation by adding an equal volume of saturated $(NH_4)_2SO_4$. The precipitate formed was centrifuged, dissolved in H_2O, and dialyzed overnight against distilled H_2O. The contents of the dialysis bag were diluted with H_2O so that 6 ml of the final

[1]Supported by the Medical Research Council of Canada and the Cooper Fund of McGill University, Montreal.

[2]The following trivial names and abbreviations are used in the text: 16-KE_2 = 16-ketoestradiol-17β (3,17β-dihydroxy-estra-1,3,5(10)-trien-16-one); estriol = 3,16α,17β-trihydroxy-estra-1,3,5(10)-triene; 16-epiestriol = 3,16β,17β-trihydroxy-estra-1,3,5(10)-triene; estrone = 3-hydroxy-estra-1,3,5(10)-trien-16-one; 16-ketoestrone = 3-hydroxy-estra-1,3,5(10)-triene-16,17-dione; dehydroisoandrosterone = 3β-hydoxy-Δ^5-androsten-17-one; NADP and NADPH = oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide phosphate; NAD and NADH = oxidized and reduced forms, respectively, of nicotinamide adenine dinucleotide; S.A. = specific activity.

preparation was equivalent to 4 ml of original whole blood. Preparations of cat erythrocytes were obtained in a similar manner, except that the material precipitating between 33% and 83% salt saturation was used for incubation.

All incubations were performed at 37 °C with 95% O_2 – 5% CO_2 or atmospheric air as the gas phase. Labelled steroid, diluted with unlabelled carrier to provide weight, was dissolved in 1–2 drops of propylene glycol and incubated with varying volumes (up to 6 ml) of enzyme preparation and 1.0 ml of 0.15 *M* phosphate buffer, pH 7.4, containing 1.5 μmoles of cofactor. The total volume was adjusted, where necessary, with H_2O to 7 ml and the time of incubation varied up to 2.5 hours. Suitable blank experiments were carried out simultaneously. Incubation mixtures were cooled, diluted with H_2O, and extracted with diethyl ether. The extracts were washed with $NaHCO_3$ solution and with H_2O, and then dried over anhydrous Na_2SO_4 before separation into ketonic and non-ketonic fractions by a modified Girard reaction (2). Radioactivity was measured by liquid scintillation spectrometry as previously described (2).

Identification of radioactive products was made by gas–liquid chromatography and by isotope dilution techniques.

Results

It can be seen from Table I that a consistent recovery of radioactivity was obtained regardless of the substrate employed and that the presence of the

TABLE I

Recovery of C^{14}-labelled substrates after incubation*

Incubation medium	Substrate	No. expts.	Recovered radioactivity (% original radioactivity)		
			Ketonic	Non-ketonic	Average total (range)
H_2O	16-KE_2-17β	1	93.6	1.5	95.1
	16-Epiestriol	1	1.8	88.7	90.5
	Estriol	1	1.9	90.9	92.8
Rat enzyme (no cofactor)	16-KE_2-17β	3	82.5	1.6	84.1 (79.5–88.4)
	16-Epiestriol	3	1.6	93.3	94.9 (90.6–99.0)
	Estriol	1	1.8	90.8	92.6
Rat enzyme (cofactor)	16-KE_2-17β	24	—	—	89.3 (73.9–99.2)
	16-Epiestriol	21	—	—	90.1 (81.1–96.5)
	Estriol	2	—	—	90.6 (89.2–92.0)
Cat enzyme (no cofactor)	16-KE_2-17β	1	84.2	2.7	86.9
	16-Epiestriol	1	1.9	91.7	93.6
	Estriol	1	2.1	92.5	94.6
Cat enzyme (cofactor)	16-KE_2-17β	20	—	—	89.6 (82.7–100)
	16-Epiestriol	2	—	—	98.7 (98.0–99.3)
	Estriol	12	—	—	92.5 (77.4–103)

*In the H_2O and enzyme blank (no cofactor) experiments incubation was for 2 hours. In the reaction mixtures with cofactor, incubation was for 0.5 to 2.5 hours with 0.5 to 6 ml of enzyme preparation. In all cases the amount of substrate was 0.1 μmole (ca. 10^4 c.p.m.) and the temperature was 37°.

enzyme fraction did not adversely influence the efficiency of the Girard separation.

Table II demonstrates the conversions catalyzed by the enzyme preparations and also the requirements for NADP or NADPH. The rat preparation did not contain NADP-dependent 16α dehydrogenase activity with estriol as substrate

TABLE II

Effect of cofactors on C^{14}-labelled substrate conversion by enzyme preparations*

Substrate	Cofactor	Substrate conversion into ketonic or non-ketonic products† (% substrate radioactivity converted)	
		Rat preparation	Cat preparation
16-Ketoestradiol-17β	NADPH	29.9	30.0
16-Ketoestradiol-17β	NADH	0.3	0.3
16-Epiestriol	NADP	73.7	0.0
16-Epiestriol	NAD	0.8	0.0
Estriol	NADP	0.0	9.7
Estriol	NAD	0.3	0.5
Estrone	NADPH	7.4	0.0
Dehydroisoandrosterone	NADPH	55.9	1.1

*Each incubation mixture consisted of 6 ml of enzyme preparation and 1 ml of 0.15 *M* phosphate buffer (pH 7.4), together with 0.1 μmole (ca. 10^4 c.p.m.) of substrate and 1.5 μmoles of cofactor. Incubation was at 37° for 2 hours.
†Each value is the average for at least two experiments and is corrected for the experimental blank (see Table I).

but did catalyze the reduction of dehydroisoandrosterone and estrone in the presence of NADPH. The cat system was devoid of 16β dehydrogenase activity when 16-epiestriol was the substrate; it did not reduce estrone and its activity towards dehydroisoandrosterone was minimal.

For both preparations increasing the enzyme concentration resulted in an approximately linear increase in activity. When the time of incubation was increased the activity also rose, but in a nonlinear fashion.

Gas–liquid chromatography of acetylated ketonic fractions obtained through enzymic dehydrogenation of the triols showed a main symmetrical peak of retention time (9.6 minutes) equal to that of 16-KE_2 diacetate. Smaller unidentified peaks were also noted. A ketonic fraction containing 17,500 c.p.m., formed by the dehydrogenation of 16-epiestriol by the rat preparation, was diluted with 140 μg of unlabelled carrier 16-KE_2 (calculated S.A. = 125 c.p.m./μg). After Celite column partition (5) the average S.A. over seven collected fractions was equal to 100 c.p.m./μg (range = 93–112 c.p.m./μg). Carrier steroid weight was measured by the Ittrich spectrophotometric method (7). Further elution of the column with methanol yielded additional unidentified radioactivity (= 2800 c.p.m.). Chromatography of the eluted 16-KE_2 on Whatman No. 42 paper in toluene – propylene glycol showed all of the radioactivity to be associated with the carrier. After elution S.A. was equal to 93 c.p.m./μg. These results suggest 16-KE_2 to be the main but not sole component of the ketonic fraction. Table III contains data on the purification of the non-ketonic fractions formed by reduction of 16-KE_2. 16-Epiestriol was the

TABLE III

Purification of non-ketonic fractions obtained by enzyme reduction of 16-KE_2-16-C^{14}*

Non-ketonic fraction (rat)		Non-ketonic fraction (cat)	
Purification step	Specific activity (c.p.m. per mg)	Purification step	Specific activity (c.p.m. per mg)
Non-ketonic fraction (12,420 c.p.m.) + 44.5 mg carrier 16-epiestriol	280 (calculated)	Non-ketonic fraction (6,680 c.p.m.) + 10.7 mg carrier estriol	624 (calculated)
Crystals	271	Crystals	580
Mother liquor	284	Mother liquor	632
Acetylation		Acetylation	
Crystals 1	274	Crystals	575
Mother liquor 1	272	Mother liquor	567
Crystals 2	275		
Mother liquor 2	279		

*Crystallization of free estrogens was done from methanol and of acetates from methanol–H_2O. Specific activities of the acetates were calculated in terms of the free estrogen.

sole product in the case of the rat preparation and estriol accounted for some 92% of the non-ketonic fraction when the cat preparation was employed.

The results in this note provide mainly qualitative information, since different enzyme preparations obtained over a period of some months have shown a quantitative variation in activity. In contrast to the behavior of salt-fractionated enzyme preparations, non-ketonic metabolites formed through the reduction of 16-KE_2 by whole erythrocytes included considerable amounts of unidentified material (2). In the present instance the identity of the ketonic compound (or compounds), formed in addition to 16-KE_2 from the triols, is not known. It could perhaps have arisen through further dehydrogenation to 16-ketoestrone, which has been claimed to be a substrate for, as well as a product of, erythrocyte dehydrogenase enzymes (8, 9). The preparations described in this paper appear to represent good examples of NADP-dependent 16α and 16β hydroxysteroid dehydrogenases of mammalian origin. Their occurrence in cat and rat erythrocytes is interesting from the point of view of comparative biochemistry. Further purification could lead to an opportunity to study stereochemical steroid–enzyme relationships with particular reference to carbon-16 of the estrogen molecule, in the general manner described by Langer *et al.* (10) for the estradiol-17β dehydrogenase of human placenta.

Acknowledgments

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Ring D–α-Ketols as Metabolic Products of Estrogen in the Nonpregnant Human[1]

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ABSTRACT. Estriol, ring D–α-ketols and estrone were measured in the urine of 9 normal males aged 17–50 yr, and 12 postmenopausal females, aged 47–72 yr. Mean values (μg/24 hr) with ranges were as follows: Males: estriol, 8.3 (3.9–13.5); ring D–α-ketols, 5.6 (2.3–10.1); estrone, 5.0 (1.5–8.3). Females: estriol, 6.0 (1.5–14.9); ring D–α-ketols, 2.8 (1.3–4.2); estrone, 2.2 (0.4–4.9). The ring D–α-ketolic fraction appears to consist, on the average, of approximately equal amounts of 16α-hydroxyestrone and a 16-ketoestradiol-17β(16-ketoestradiol) fraction perhaps containing some 16β-hydroxyestrone. Analysis of C^{14}–ring D–α-ketols from the urine of 3 subjects given estrone-16-C^{14} suggests that 16α-hydroxyestrone and the 16-ketoestradiol fraction arise from estrone, perhaps *via* some common intermediate. No proof could be obtained for the conversion *in vivo* of 16-ketoestradiol to 16α-hydroxyestrone, either directly or *via* the triols.

RING D–α-ketolic estrogens such as 16α-hydroxyestrone,[2] 16-ketoestradiol-17β (16-ketoestradiol) and 16β-hydroxyestrone have been identified both as urinary excretion products in human pregnancy (1, 2) and as metabolites of C^{14}-labeled estrogens in the nonpregnant human (3, 4). Investigations performed *in vivo* and *in vitro* with human liver tissue have shown these same compounds to be important precursors of the various isomeric estriols (5–8). Ring D–α-ketols may perhaps be formed from estrone (or estradiol-17β) *via* an intermediate such as 16-ketoestrone (9).

A previous communication from this laboratory (10) showed the ring D–α-ketols to be quantitatively important metabolites in human pregnancy urine. However, little appears to have been reported on these compounds as endogenous urinary metabolites in the nonpregnant human. The present report is concerned with this latter aspect of the problem. In addition, certain studies are described regarding the transformation of C^{14}-labeled estrogens to ring D–α-ketols, thus yielding information on the mode of production of the latter. The experiments described below on the metabolism of injected estrone-16-C^{14}, 16-ketoestradiol-16-C^{14} and 16-*epi*estriol-16-C^{14} have already been reported by others (4, 6). These have been repeated here, using the same methodology as for measurement of endogenous urinary ring D–α-ketols so that the two types of experiment could be more closely compared.

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[1] Presented in part at the Fourth Annual Meeting of the Canadian Federation of Biological Societies, Guelph, Ontario, June, 1961.

[2] The following trivial names are used in the text: Estrone = 3-hydroxy-1,3,5(10)-estratriene-16-one; estradiol-17β = 3,17β-dihydroxy-1,3,5(10)-estratriene; estriol = 3,16α,17β-trihydroxy-1,3,5(10)-estratriene; 16-*epi*estriol = 3,16β,17β-trihydroxy-1,3,5(10)-estratriene; 16α-hydroxyestrone = 3,16α-dihydroxy-1,3,5(10)-estratriene-17-one; 16β-hydroxyestrone = 3,16β-dihydroxy-1,3,5(10)-estratriene-17-one; 16-ketoestradiol-17β = 3,17β-dihydroxy-1,3,5(10)-estratriene-16-one; 16-ketoestrone = 3-hydroxy-1,3,5(10)-estratriene-16,17-dione; 2-methoxyestrone = 2-methoxy-3-hydroxy-1,3,5(10)-estratriene-16-one.

TABLE 1. Excretion of radioactivity by five subjects following injection of C^{14}-estrogens

Subject	Age (yr)	Material injected			Days of urine collection	Total radioactivity in urine counts/min $\times 10^6$	Total activity as % of dose
		C^{14}-estrogen	μg	counts/min $\times 10^6$			
A, male	45	estrone	47.4	0.865	4	0.508	59
B, female	52	estrone	75.6	1.38	4	0.938	64
C, female	50	estrone	31.6	0.577	1	—	—
D, male	60	16-ketoestradiol	47.0	0.70	2	0.381	54
E, male	62	16-*epi*estriol	47.2	0.519	4	0.446	76

Materials and Methods

Subjects. Measurement of endogenous estrogen metabolites was performed on 12 postmenopausal women and 9 males, all 21 of whom exhibited normal liver function by at least 2 biochemical tests. The metabolism of C^{14}-estrogens was studied in 5 subjects (2 postmenopausal females and 3 males) who also had normal liver function.

Measurement of Endogenous Estrogen Metabolites. Upon completion of collection, each 24-hr urine was diluted to 2,500 ml with distilled water, and quadruplicate 50 ml volumes were incubated with 20 units of bacterial β-glucuronidase[3] per ml for 24 hr at pH 6.5 and 37 C (11). One duplicate pair was analyzed for estriol and estrone by the method of Bauld (12), using the fluorimetric technique described by Ittrich (13) for the final measurement. Tetrabromoethane was used as solvent. The other duplicate pair was analyzed for the ring D–α-ketolic estrogen fraction by the procedure of Givner *et al.* (14), with a few modifications. The partition column described by Givner *et al.* (15) for the separation of ketonic estrogens was prepared in benzene:hexane (1:1) in place of benzene :hexane (1:49) as mobile phase; the stationary phase remained as methanol:water (7:3). The ketonic fraction from the Girard reaction was transferred to this column in 3×1 ml of mobile phase rather than by the celite transfer technique (15). The initial 40 ml of this same mobile phase used to develop the column eluted less polar ketones including estrone. Further elution with 100 ml of benzene:hexane (1:1) yielded the ring D–α-ketols. In trial experiments this resulted in an over-all increase of 10–15% in recovery of pure 16α-hydroxyestrone or 16-ketoestradiol over that described earlier (10, 14). Final measurement of the ring D–α-ketolic fraction was performed by the Ittrich fluorimetric method (13) using tetrabromoethane as solvent and 16α-hydroxyestrone as standard.

Recovery Experiments. Amounts of estriol, estrone and 16α-hydroxyestrone or 16-ketoestradiol, ranging from 0.10–0.30 μg, were added, in small amounts of ethanol, to 50 ml volumes of incubated nonpregnancy urine. Analyses were performed as described above and recoveries, corrected for urine blanks, were calculated.

Chemical Reduction of Ring D–α-Ketols. Urinary ring D–α-ketolic fractions and pure samples of 16α-hydroxyestrone and 16-ketoestradiol were reduced by treatment overnight in 50% aqueous ethanol with 10 mg/ml of sodium borohydride (1, 16). Following suitable extraction, the triols formed were separated into estriol- and 16-*epi*estriol-containing fractions by column partition (15). Measurement of these fractions was performed by the Ittrich technique (13), either fluorimetrically, using tetrabromoethane as solvent, or colorimetrically, using tetrachloroethane as solvent. Where applicable, such fractions were also submitted to radioactive counting.

Counting of C^{14}. All counting was done in the Geiger region, using a Nuclear Chicago D47 gas flow counter with thin end window. Samples were plated from ethanolic solution in cupped nickel-plated planchettes (5/16″ ×1″). Direct counts on urine samples were corrected for self-absorption by the internal standard technique employed by Engel *et al.* (11). Sufficient counts were collected to ensure an accuracy to within ±5%.

C^{14}-Estrogens. Estrone-16-C^{14} (Radiochemical

[3] Obtained from Sigma Chemical Co., St. Louis, Missouri.

TABLE 2. Urinary excretion (μg/24 hr) of endogenous estrogen metabolites by normal males

Age (yr)	Estriol	Ketols	Estrone	Total*
17	4.1	3.0	1.5	8.6
26	6.9	6.6	7.1	20.6
28	3.9	10.1	3.6	17.6
30	11.2	2.3	6.8	20.3
31	6.1	6.3	3.8	16.2
31	10.7	7.2	2.9	20.8
32	10.3	4.9	5.6	20.8
45	13.5	4.5	8.3	26.3
50	7.6	5.6	5.1	18.3
Mean (range)	8.3 (3.9–13.5)	5.6 (2.3–10.1)	5.0 (1.5–8.3)	18.8 (8.6–26.3)

* Total of the 3 fractions measured.

Centre, Amersham, England) was shown to be radiochemically homogeneous by column partition chromatography. Using the above counting technique, the specific activity was 18,000 counts/min/μg.

16-Ketoestradiol-16-C^{14}, prepared by Levitz (17), was purified by column partition (15). Further purification by paper chromatography in the system chloroform:formamide (2) yielded highly pure material of specific activity 14,900 counts/min/μg.

A separate portion of 16-ketoestradiol-16-C^{14} was reduced by sodium borohydride, as described above. The triols formed were saponified, re-extracted into ether and chromatographed on a partition column as described for nonketonic estrogens by Givner *et al.* (15). The 16-*epi*estriol-16-C^{14} fraction obtained had a specific activity of 11,000 counts/min/μg and was radiochemically homogeneous when chromatographed on paper in the systems benzene:hexane:methanol:water (4:1:4:1) and chloroform:formamide.

Administration of C^{14}-Estrogens. These were administered intravenously in a small volume of propylene glycol. Vials and syringes were washed out with ethanol and the contents were analyzed to correct for losses of the original material. Estrone-16-C^{14} was given to 1 male (subject A) and 2 females (subjects B and C) while 16-ketoestradiol-16-C^{14} and 16-*epi*estriol-16-C^{14} were each given to 1 male (subjects D and E, respectively). Table 1 contains data on dosage.

Analysis of Urinary C^{14}-Metabolites. Total counts were determined on all urines (Table 1), and, depending on the magnitude of these, one-half aliquots or complete 24-hr volumes were hydrolyzed by bacterial enzyme, as described above. In the experiments involving estrone-16-C^{14}, 6 radioactive urinary estrogen fractions were separated by the

TABLE 3. Urinary excretion (μg/24 hr) of endogenous estrogen metabolites by postmenopausal females

Age (yr)	Estriol	Ketols	Estrone	Total*
47	14.9	3.9	2.7	21.5
52	3.0	3.5	2.2	8.7
55	11.5	3.0	1.2	15.7
60	5.1	3.5	4.2	12.8
60	4.1	3.2	4.9	12.2
60	1.5	3.0	0.6	5.1
66	6.8	4.2	4.4	15.4
66	4.3	1.4	1.6	7.3
67	3.0	2.7	0.7	6.4
69	3.7	1.3	0.6	5.6
71	10.8	1.9	3.1	15.8
72	2.9	1.8	0.4	5.1
Mean (range)	6.0 (1.5–14.9)	2.8 (1.3–4.2)	2.2 (0.4–4.9)	11.0 (5.1–21.5)

* Total of the 3 fractions measured.

TABLE 4. Borohydride reduction products of pure 16α-hydroxyestrone and 16-ketoestradiol

Starting material	Estriol fraction		16-*epi*Estriol fraction		Total	
	μg	% recovery	μg	% recovery	μg	% recovery
6α-Hydroxyestrone 108 μg	71.0	65.8	11.8*	10.9	82.8	76.7
16-Ketoestradiol 100 μg	4.7	4.7	89.6	89.6	94.3	94.3

* The reduction product of 16α-hydroxyestrone appearing in the 16-*epi*estriol fraction is probably 17-*epi*estriol (1).

method of Givner *et al.* (14). In the 16-ketoestradiol-16-C^{14} experiment, only the ring D–α-ketolic fraction was analyzed and, after the administration of 16-*epi*estriol-16-C^{14}, the fractions containing the ring D–α-ketols, 16-*epi*estriol and estriol were each collected and studied. In all cases the ring D–α-ketolic fractions (with or without added carrier steroids) were reduced with sodium borohydride (see above), and the triols formed chromatographed on partition columns (15) to yield 16-*epi*estriol and estriol fractions. These latter were analyzed by the Ittrich fluorimetric or colorimetric method, and counted; from these data their specific activities were determined.

Results

Tables 2 and 3 contain data on the levels of endogenous estriol, estrone and ring D–α-ketols in the urine of males and postmenopausal females, respectively. On the average, the ring D–α-ketols were similar to estrone, although there were variations from one individual to another. It should be noted that the results in these tables are uncorrected for experimental losses. In trial experiments, recovery values for pure compounds have been: estriol, 78–93%; estrone, 85–95%; 16α-hydroxyestrone or 16-ketoestradiol, 64–80%. The reproducibility of the analytic procedure for the concentration ranges measured (Tables 2 and 3) was obtained by calculating the standard deviation of the difference between duplicate analyses for the three urinary fractions. For estriol this value was 0.37 μg, for estrone 0.30 μg, and for the ring D–α-ketols 0.69 μg. A urinary ring D–α-ketolic fraction, prepared by pooling small portions of urinary fractions left over following fluorimetry, was reduced with sodium borohydride under the usual conditions. It was found that 0.9 μg of original ring D–α-ketols (in terms of 16-ketoestradiol) yielded 0.30 μg of estriol and 0.33 μg of 16-*epi*estriol. The apparent loss of material may be partly accounted for by experimental losses, as shown in Table 4 for the chromatographic separation of the borohydride reduction products of authentic 16α-

TABLE 5. Radioactive fractions in the urine of subjects given C^{14}-estrogens

Subject*	Estrogen injected	Radioactivity in fractions as % of dose						
		Estriol	Ketols	Estrone	Estradiol	16-*epi*-Estriol	2-Methoxy-estrone	Total†
A	estrone	9.7	6.1	7.0	2.6	2.6	1.3	29.3
B	estrone	6.6	7.4	8.3	1.9	2.0	1.7	27.9
D‡	16-ketoestradiol	—	13.2	—	—	—	—	—
E‡	16-*epi*estriol	3.5	0.8	—	—	11.9	—	16.2

* For further details see Table 1.
† Total refers to sum of fractions shown.
‡ Only the fractions shown above to contain radioactivity were measured in these subjects.

TABLE 6. Analysis of borohydride reduction products of urinary C^{14}–ring D–α-ketols following administration of estrone-16-C^{14} to subject A*

Days following C^{14}-estrone	Estriol fraction†			16-*epi*Estriol fraction‡		
	μg	counts/min	counts/min/μg	μg	counts/min	counts/min/μg
1	2.0	4,000	2,000	1.6	3,800	2,380
2	0.8	3,200	4,000	0.9	3,900	4,340
3	0.7	440	630	1.0	850	850
4	0.8	260	330	1.2	490	410
Total	4.3	7,900	—	4.7	9,040	—
Mean specific activity§	—	—	1,840	—	—	1,920

* For further details see Tables 1 and 5.
† Main reduction product of 16α-hydroxyestrone (see Table 4).
‡ Main reduction product of 16-ketoestradiol (see Table 4).
§ Calculated from total weights and counts over 4 days.

hydroxyestrone and 16-ketoestradiol.

Table 5 shows the pattern of six metabolites of estrone-16-C^{14} in two subjects in terms of radioactivity of the fractions obtained from the partition columns. This table also contains data on the excretion of some radioactive fractions following the administration of 16-ketoestradiol-16-C^{14} and 16-*epi*estriol-16-C^{14}.

Tables 6 and 7 contain information on the borohydride reduction products of the urinary ring D–α-ketols (no unlabeled carrier added) formed from estrone-16-C^{14} in subjects A and B. In both cases the specific activities of the estriol and 16-*epi*estriol fractions (reflecting those of 16α-hydroxyestrone and 16-ketoestradiol, respectively; see Table 4) were similar. Moreover, the weights of both metabolites were also very similar over the four-day study period. Only one 24-hour urine could be obtained from subject C following estrone-16-C^{14} injection. The ring D–α-ketolic fraction yielded, on reduction, 2.2 μg of estriol containing 3,000 cpm (specific activity = 1,360 counts/min/μg) and 2.7 μg of 16-*epi*estriol containing 4,300 cpm (specific activity = 1,590 counts/min/μg).

In Table 8, results of specific activity measurements are given for the reduction products of urinary ring D–α-ketols following administration of 16-ketoestradiol-16-C^{14} to subject D and 16-*epi*estriol-16-C^{14} to subject E. In these

TABLE 7. Analysis of borohydride reduction products of urinary C^{14}–ring D–α-ketols following administration of estrone-16-C^{14} to subject B*

Days following C^{14}-estrone	Estriol fraction†			16-*epi*Estriol fraction‡		
	μg	counts/min	counts/min/μg	μg	counts/min	counts/min/μg
1	0.7	3,500	5,000	1.1	4,700	4,270
2	1.4	8,600	6,150	2.1	10,100	4,800
3	1.6	4,700	2,940	1.9	5,100	2,680
4	3.8	3,900	1,030	3.2	2,300	720
Total	7.5	20,700	—	8.3	22,200	—
Mean specific activity§	—	—	2,760	—	—	2,680

* For further details see Tables 1 and 5.
† Main reduction product of 16α-hydroxyestrone (see Table 4).
‡ Main reduction product of 16-ketoestradiol (see Table 4).
§ Calculated from total weights and counts over 4 days.

TABLE 8. Activities of borohydride reduction products of urinary C^{14}-ring D-α-ketols following administration of C^{14}-estrogens to subjects D and E*

Estrogen† injected	Estriol fraction‡			16-*epi*Estriol fraction§		
	μg	counts/min	counts/min/μg	μg	counts/min	counts/min/μg
16-Ketoestradiol	82.8	510	6.2	98.0	6,980	71.3
16-*epi*Estriol	75.2	360	4.8	90.2	2,870	31.8

* Prior to reduction of the urinary ring D-α-ketols, 108 μg of pure 16α-hydroxyestrone and 100 μg of pure 16-ketoestradiol (both unlabeled carriers) were added.
† For further details of amounts injected and of radioactive urinary fractions, see Tables 1 and 5.
‡ Main reduction product of 16α-hydroxyestrone (see Table 4).
§ Main reduction production of 16-ketoestradiol (see Table 4).

instances, known amounts of pure carrier 16α-hydroxyestrone and 16-ketoestradiol had been added to the ring D-α-ketolic fraction prior to reduction. These data indicate that little, if any, 16α-hydroxyestrone could have been excreted in the urine as a metabolite of 16-ketoestradiol or 16-*epi*estriol. In order to obtain additional proof that C^{14}-estriol was a true urinary metabolite, following administration of 16-*epi*estriol-17-C^{14} to subject E (Table 5), 10 mg of pure crystalline carrier estriol was added to part of the radioactive estriol fraction (9,000 counts/min) from the column and recrystallized three times from methanol. Crystals and mother liquors were analyzed at each stage and specific activities were determined. Crystals 1, 2 and 3 had specific activities of 760, 720 and 734 counts/min/mg, respectively, and the corresponding mother liquors measured 700, 760 and 765 counts/min/mg. This indicated production of C^{14}-estriol from 16-*epi*estriol-16-C^{14}, presumably *via* 16-ketoestradiol.

Discussion

Initial attempts to measure urinary ring D-α-ketols in nonpregnant humans by the procedure of Givner *et al.* (14) were unsuccessful in this laboratory. This was primarily due to background color produced by urinary residues in the Bauld modification of the Kober reaction (18). The combination of techniques described in this paper, including fluorimetric analysis, has yielded results which are considered to be meaningful.

In both males and postmenopausal females, the levels of ring D-α-ketols, on the average, are very similar to those of estrone and less than estriol (Tables 2 and 3). A few points concerning possible inaccuracies in the measurement of these ketols should, however, be considered. First of all, these compounds were measured in terms of 16α-hydroxyestrone, which, in the author's hands, has a lower fluorescence intensity than does 16-ketoestradiol (ratio 0.83:1.0). This would result in the overestimation of total ketols. On the other hand, however, the recovery of the ketols is lower than that of estrone (see Results), so that the relative levels given in the tables may not be much in error. A second, and perhaps more serious question, concerns the hydrolytic cleavage of the conjugated ring D-α-ketols. It is difficult to compare results obtained by enzymic hydrolysis with any standard procedure, since the ketols are unstable to hot acid treatment. If the urinary ketols in the nonpregnant state are conjugated to some extent as sulfates, the levels given here will be low, since the bacterial β-glucuronidase preparation does not contain sulfatase activity (19, 20). Unpublished results from this laboratory show that this bacterial enzyme preparation is an excellent tool for splitting conjugated estriol, estrone and estradiol in the urine of nonpregnant humans. This supports the data reported by Engel *et al.* (11).

When considered in the light of their

borohydride reduction products, the urinary ring D–α-ketolic fractions appear to consist of approximately equal amounts of 16α-hydroxyestrone and a fraction containing 16-ketoestradiol and perhaps 16β-hydroxyestrone. It should be noted that 16β-hydroxyestrone as well as 16-ketoestradiol would give rise mainly to 16-*epi*estriol on reduction with borohydride. On the basis of the present study, a choice between these two compounds is not possible. If previous results on pregnancy urine (2, 10) are any criteria, 16-ketoestradiol is likely to be quantitatively more significant than 16β-hydroxyestrone. The specific activities of the chemically reduced ring D–α-ketols formed following injection ot estrone-16-C^{14} to subjects D and E (Tables 6 and 7) are in agreement with the thesis that these ketols arise from estrone, perhaps *via* some common intermediate such as 16-ketoestrone (9). The possibility that 16α-hydroxyestrone is readily formed from 16-ketoestradiol, either directly or *via* the triols, is unlikely in view of the studies made by Levitz *et al.* (6). In the present instance it is difficult to rule out the production of a small amount of 16α-hydroxyestrone from these sources, since 16-ketoestradiol, when reduced, always gives rise to a small amount of material in the estriol fraction (see Table 4). Although 16-ketoestradiol is a metabolite of the triols (ref. 2 and Table 5), this conversion may be of minor quantitative significance when compared with its formation from estrone (Table 5). This question will not be settled until the urinary 16-ketoestradiol and 16β-hydroxyestrone are separated and individually measured.

It is of interest to note that if the ring D–α-ketols in males and postmenopausal females (Tables 2 and 3) are calculated as a percentage of estriol, ketols and estrone, the average values are 30% for males and 25% for females. A similar calculation for the radioactivity excreted in these fractions following estrone-16-C^{14} injection in subjects A and B (Table 5) gives values of 27 and 33%, respectively. Thus, by two different criteria the patterns of estriol, ring D–α-ketols and estrone are similar.

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PERIPHERAL INTERCONVERSION OF PHENOLIC STEROIDS IN THE HUMAN[1]

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Abstract

A tracer dose of estradiol-17β-6,7-^{3}H was injected intravenously into male subjects simultaneously with a ^{14}C-labeled steroid. The latter was either 16-ketoestradiol-17β-16-^{14}C, 16-epiestriol-16-^{14}C, or estriol-16-^{14}C. All eight urinary metabolites of estradiol-17β-^{3}H studied, and the main metabolites of the 16-oxygenated steroids, were predominantly split by β-glucuronidase hydrolysis. The major urinary metabolite released by solvolysis was estrone, as a conversion product of estradiol-17β-^{3}H. After injection of estradiol-17β-^{3}H together with either 16-ketoestradiol-17β-^{14}C or 16-epiestriol-^{14}C, urinary 16β-hydroxyestrone contained both isotopes. 16α-Hydroxyestrone, on the other hand, contained ^{3}H but only insignificant amounts of ^{14}C. This suggests formation of 16β-hydroxyestrone but not 16α-hydroxyestrone from these injected 16-oxygenated steroids. 16-Epiestriol was metabolized to identifiable urinary metabolites to a considerably greater degree than was estriol. This was confirmed in a further experiment in which only 16-epiestriol-^{14}C was injected.

Introduction

Considerable knowledge concerning the nature of the peripheral interconversion of phenolic steroids in the human has become available during the past decade (1). Contributing largely to this has been the isolation of a number of hitherto unknown urinary metabolites. Among the latter are several 16-oxygenated steroids such as 16-epiestriol[2] (2), 16α-hydroxyestrone (3), 16β-hydroxyestrone (4), and 16-ketoestradiol-17β (4) (hereafter referred to as 16-ketoestradiol) whose interrelationships have been at least partially resolved (5–8). Gallagher *et al.*, in a series of publications reviewed by Brown (9), established the general pattern of these compounds, and others, as urinary metabolites of injected estradiol-17β (hereafter referred to as estradiol) in the human. However, very few studies have been published in which more than the three 'classical' estrogens (estrone, estradiol, and estriol) were investigated in one and the same subject (10, 11). In the present instance, the production of some 16-oxygenated phenolic steroids has been studied following simultaneous injection of estradiol-6,7-^{3}H and certain ^{14}C-labeled 16-oxygenated steroids. In addition, some information has been obtained regarding the mode of conjugation of eight urinary estrogen metabolites.

[1]Supported in part by a grant from the Medical Research Council of Canada.

[2]The following abbreviations and trivial names are used in the text: 16-epiE_3 = 16-epiestriol (3,16β,17β-trihydroxy-estra-1,3,5 (10)-triene); 16-KE_2 = 16-ketoestradiol-17β (3,17β-dihydroxy-estra-1,3,5 (10)-trien-16-one); 16α-OHE_1 = 16α-hydroxyestrone (3,16α-dihydroxy-estra-1,3,5 (10)-trien-17-one); 16β-OHE_1 = 16β-hydroxyestrone (3,16β-dihydroxy-estra-1,3,5 (10)-trien-17-one); E_3 = estriol (3,16α,17β-trihydroxy-estra-1,3,5 (10)-triene); 16-ketoestrone = 3-hydroxy-estra-1,3,5 (10)-triene-16,17-dione; E_1 = estrone (3-hydroxy-estra-1,3,5 (10)-trien-17-one); E_2 = estradiol-17β (3,17β-dihydroxy-estra-1,3,5 (10)-triene); 2-MeE_1 = 2-methoxyestrone (2-methoxy-3-hydroxy-estra-1,3,5 (10)-trien-17-one); c.p.m. = counts per minute; SA = specific activity; G = urinary steroid conjugate fraction split by β-glucuronidase; S = urinary steroid conjugate fraction split by solvolysis.

Materials and Methods

Radioactive Steroids

Estradiol-6,7-^{3}H of specific activity (SA) 40 μcuries/μg was purchased from Merck Sharpe & Dohme and purified by Celite column partition chromatography (12). The material collected in the peak tubes was pure as judged by crystallization with unlabeled carrier. 16-Ketoestradiol-16-^{14}C[3] (SA = 44 μcuries/mg) was obtained in an impure state. It was submitted to a modified Girard separation (12) and the ketonic fraction was treated by thin-layer chromatography (13). The eluted radioactivity was chromatographed on a Celite partition column and then further purified by paper chromatography in toluene – propylene glycol. The material so obtained was shown to be at least 98.5% pure by crystallization with unlabeled carrier steroid. 16-Epiestriol-16-^{14}C was prepared from the latter purified 16-ketoestradiol by reduction with sodium borohydride and subsequent column partition chromatography (12). After thin-layer chromatography and elution, the labeled material was found to be at least 98% pure by crystallization with unlabeled carrier steroid. Estriol-16-^{14}C[3] (SA = 22 μcuries/mg) was chromatographed on paper and a partition column and shown to be at least 99% pure by crystallization with unlabeled carrier.

Non-Radioactive Steroids

2-Methoxyestrone and 16-epiestriol were purchased from Organon Laboratories Ltd.; estrone and estradiol from Mann Research Laboratories Inc., New York; 16-ketoestradiol from Steraloids Inc., Pawling, New York; and 16β-hydroxyestrone diacetate from Southeastern Biochemicals Inc., Morristown, Tennessee. 16α-Hydroxyestrone[4] and estriol[5] were donated. Where necessary these were crystallized to yield melting points which agreed closely with the theoretical.

Chemicals and Reagents

Pyridine and acetic anhydride were fresh anhydrous materials. All other reagents and solvents were of good grade and were purified where necessary by standard procedures.

Administration of Radioactive Steroids

This was done via an arm vein as a single dose in 10 ml of 10% ethanol in saline. Vials and syringes were washed out following injection and the activity remaining was counted so that the absolute dose could be calculated. The subjects injected were four males with no endocrine or liver abnormalities.

Urine Collection and Analysis

Urine was collected in each case for 96 hours after injection and frozen in polyethylene bottles until required. Aliquots of, or total 96-hour pooled urines

[3]Generously donated by Dr. M. Levitz, New York Medical Centre.
[4]Generously donated by Dr. A. E. Kellie, Middlesex Hospital, London, England.
[5]Generously donated by Parke Davis & Company, Ltd.

were extracted serially following β-glucuronidase incubation (14) and solvolysis (15). These extracts correspond approximately to glucosiduronate and sulfate conjugates, respectively, and are referred to as G and S fractions. Each extract was submitted to a modified Girard separation and the ketonic fractions obtained were chromatographed on Celite partition columns (16). The corresponding non-ketonic fractions were further purified by alkaline treatment and then subjected to column partition (16).

Ring D α-ketolic fractions were chromatographed on Whatman No. 42 paper in toluene – propylene glycol at 23° for 4 days against appropriate standards. Under these conditions, 16β-hydroxyestrone traveled 13 cm, 16α-hydroxyestrone 23 cm, and 16-ketoestradiol 35 cm. The separated compounds were eluted with ethyl acetate, the extracts washed with H_2O, and evaporated. Where insufficient unlabeled 16α-hydroxyestrone was available for use as carrier (experiment 3) in subsequent isotope dilution, the fraction eluted from the paper was reduced with sodium borohydride to yield mainly estriol (14) and this was purified as the triol. In all cases 16β-hydroxyestrone was acetylated immediately after elution from the paper and purified as the diacetate.

Eluted material from the columns and papers, containing known amounts of radioactivity, was diluted with accurately weighed amounts (10–30 mg) of appropriate unlabeled carrier steroids. This was followed by at least two crystallizations (except for 16β-hydroxyestrone and in some cases 16-ketoestradiol) as the free compounds, from methanol. The crystals were then acetylated overnight at room temperature in a mixture of 0.2 ml anhydrous pyridine and 0.1 ml acetic anhydride. The acetates so formed were crystallized at least twice from methanol–water mixtures. Crystallization was continued as far as possible until successive SA values differed by no more than 5% and, where appropriate, ^3H:^{14}C ratios became constant. Melting point determinations (microscope hot stage) helped to confirm the identity and purity of the final products.

All radioactive counting was performed on a Nuclear-Chicago model 6725 liquid-scintillation spectrometer using the formula of Okita *et al.* (17) to calculate ^3H and ^{14}C counts where necessary. Efficiencies of counting for these isotopes were 25% and 60% respectively. Under these conditions the contribution of ^3H to the ^{14}C channel was less than 1% and that of ^{14}C to the ^3H channel approximately 15%. The background count in each channel was about 30 c.p.m. The scintillation medium consisted of toluene containing 0.3% (w/v) of 2,5-triphenyloxazole and 0.01% (w/v) of 1,4-bis-2-(5-phenyloxazolyl)-benzene.

Results

Table I shows the radioactivity and ^3H:^{14}C ratios of the injected steroids and the recovery of these in the urinary extracts. Most of the extractable activity had apparently been conjugated as glucosiduronate with small but significant amounts, particularly as metabolites of estradiol, being released by solvolysis.

TABLE I

Injection and urinary excretion of radioactive steroids in male subjects

	Experiment No.			
	1	2	3	4
Steroids injected	E_2-^3H + 16-KE_2-^{14}C	E_2-^3H + 16-epiE_3-^{14}C	E_2-^3H + E_3-^{14}C	16-epiE_3-^{14}C
^3H injected (c.p.m.)	9.4×10^6	1.06×10^7	6.6×10^6	—
^{14}C injected (c.p.m.)	1.02×10^6	1.25×10^6	1.64×10^6	0.24×10^6
^3H:^{14}C injected	9.2	8.5	4.0	—
^3H (% dose) in urine extracts*				
G	35.3	47.2	28.8	—
S	6.5	5.7	2.2	—
^{14}C (% dose) in urine extracts*				
G	43.4	66.6	42.7	41.5
S	Trace	Trace	2.6	†

*After hydrolysis, extraction, and Girard separation.
†Not done.

As expected, estrone, estradiol, and 2-methoxyestrone were detected in the urine after injection of estradiol-^3H in experiments 1–3. Identity was proved in each case by crystallization to constant specific activity with the appropriate carrier steroid. Detailed data are not shown for the purification of these compounds.

Tables II–IV contain detailed information on the purification of 16-oxygenated metabolites of injected estradiol-^3H together with a ^{14}C-labeled 16-oxygenated steroid in experiments 1–3. When 16-ketoestradiol was the ^{14}C-labeled compound (Table II), all of the 16-oxygenated metabolites analyzed, with the exception of 16α-hydroxyestrone, possessed ^3H:^{14}C ratios considerably lower than that of the injected mixture. The amount of ^{14}C associated with 16α-hydroxyestrone was so small as to be insignificant. In the case of injected estradiol-^3H plus 16-epiestriol-^{14}C (Table III), none of the latter isotope was incorporated into 16α-hydroxyestrone. The other four metabolites were labeled with both isotopes, with 16β-hydroxyestrone and 16-ketoestradiol in particular formed from injected 16-epiestriol to a greater extent than from estradiol. In experiment 3 (Table IV) estriol was not significantly metabolized to identifiable products. It should be noted that although the 16-ketoestradiol fraction, after paper chromatography and crystallization as the free compound, contained considerable radioactivity, little remained after crystallization as the diacetate. Also, although the purified 16α-hydroxyestrone had a ^3H:^{14}C ratio of 13, the contribution of ^{14}C was so small as to be insignificant.

Table V shows the purification of three metabolites after injection of 16-epiestriol-^{14}C alone. The results bear out those of experiment 2 (Table III) indicating a significant conversion to 16-ketoestradiol and estriol.

The conversion of injected estradiol-^3H to urinary metabolites is shown in Table VI. These figures were calculated as percentage of dose from the mathematical product of the final specific activity of the purified crystals and the

TABLE II

Purification of urinary 16-oxygenated metabolites following injection of E_2-^3H + 16-KE_2-^{14}C (^3H:^{14}C ratio = 9.2; experiment 1*)

Isotope	Purification† step	16α-OHE_1	16β-OHE_1‡	16-KE_2‡		16-epiE_3		E_3	
		G	G	G	S	G	S	G	S
^3H	SA, calculated	4,500	1,500	1,860	1,550	3,100	890	13,300	2,900
	SA, crystals 1	3,300	850	1,430	860	2,500	550	—	—
	2	2,400	790	1,360	680	2,100	520	14,400	2,700
	3	2,900	780	1,390	690	2,400	430	14,500	2,600
	4	3,000	—	—	—	2,400	500	14,000	2,500
^{14}C	SA, calculated	150	300	1,200	760	830	90	4,300	680
	SA, crystals 1	100	280	1,380	470	1,000	185	—	—
	2	30	280	1,380	400	860	170	5,000	900
	3	60	270	1,380	410	1,100	190	5,300	900
	4	44	—	—	—	1,100	190	4,900	870
Final ^3H:^{14}C ratio		68	2.9	1.0	1.7	2.2	2.6	2.9	2.9

*All SA values (c.p.m. per mg) are calculated for free steroids.
†After addition of appropriate unlabeled carrier; crystallizations 1 and 2 done on free steroids (methanol), 3 and 4 on acetates (methanol–water), except as otherwise indicated.
‡Crystallized as acetates throughout.

TABLE III

Purification of urinary 16-oxygenated metabolites following injection of E_2-^3H + 16-epiE_3-^{14}C (^3H:^{14}C ratio = 8.5; experiment 2*)

Isotope	Purification† step	16α-OHE_1	16β-OHE_1‡	16-KE_2‡	16-epiE_3		E_3	
		G	G	G	G	S	G	S
^3H	SA, calculated	1,770	950	1,100	5,000	590	52,000	980
	SA, crystals 1	1,120	520	1,060	4,100	220	51,500	790
	2	1,170	480	970	4,000	240	51,000	800
	3	—	440	870	4,200	200	52,000	800
	4	—	—	—	4,300	—	50,000	800
^{14}C	SA, calculated	—	600	520	7,500	260	4,800	60
	SA, crystals 1	—	480	580	7,300	260	4,850	60
	2	—	470	560	7,300	320	4,400	60
	3	—	440	510	8,400	330	5,000	67
	4	—	—	—	8,500	—	4,900	65
Final ^3H:^{14}C ratio		—	1.0	1.7	0.5	0.6	10.2	12.3

*All SA values (c.p.m. per mg) are calculated for free steroids.
†After addition of appropriate unlabeled carrier; crystallizations 1 and 2 done on free steroids (methanol), 3 and 4 on acetates (methanol–water), except as otherwise indicated.
‡Crystallized as acetates throughout.

TABLE IV

Purification of urinary 16-oxygenated metabolites following injection of E_2-^3H + E_3-^{14}C (^3H:^{14}C ratio = 4.0; experiment 3*)

Isotope	Purification† step	16α-OHE_1 G	16-KE_2‡ G	16-epiE_3 G	16-epiE_3 S	E_3 G	E_3 S
^3H	SA, calculated	1,040	4,000	1,570	190	3,900	570
	SA, crystals 1	510	3,500	700	56	3,840	460
	2	520	690	710	38	3,440	440
	3	530	640	740	28	3,500	430
^{14}C	SA, calculated	22	220	180	23	28,200	2,860
	SA, crystals 1	39	530	120	13	30,400	3,200
	2	43	170	122	8	29,400	3,300
	3	40	150	123	6	29,800	3,200
Final ^3H:^{14}C ratio		13	4.3	6.0	4.7	0.12	0.13

*All SA values (c.p.m. per mg) are calculated for free steroids.
†After addition of appropriate unlabeled carrier; crystallizations 1 and 2 done on free steroids (methanol), 3 on acetates (methanol–water) except as otherwise indicated.
‡Crystallization 1 done on free steroid, 2 and 3 on acetate.

TABLE V

Purification of urinary 16-oxygenated metabolites following injection of 16-epiE_3-^{14}C (experiment 4*)

Purification† step	16-KE_2 G	16-epiE_3 G	E_3 G
SA, calculated	830	1830	390
SA, crystals 1	785	1905	400
2	742	1780	440
3	790	1760	430
4	—	1680	450

*All SA values (c.p.m. per mg) are calculated for free steroids.
†After addition of appropriate carrier; crystallizations 1 and 2 done on free steroids (methanol), 3 and 4 on acetates (methanol–water).

TABLE VI

Conversion (% dose) of E_2-^3H to urinary metabolites as calculated from crystallization data

Metabolite	Expt. 1 G	Expt. 1 S	Expt. 2 G	Expt. 2 S	Expt. 3 G	Expt. 3 S
2-MeE_1	0.6	0.2	0.5	0.1	2.0	0.2
E_1	5.4	1.9	7.9	2.4	4.7	0.3
E_2	2.6	0.2	3.3	0.8	2.5	<0.1
16α-OHE_1	1.3	ca. 0.1	0.6	—	0.3	—
16β-OHE_1	0.2	—	0.4	—	—	—
16-KE_2	0.6	0.2	1.2	<0.1	0.3	—
16-epiE_3	1.1	0.1	2.0	<0.1	0.2	<0.1
E_3	6.7	0.7	13.2	0.5	1.2	0.1
Total	18.5	3.4	29.1	3.8	11.2	0.6

weight of unlabeled carrier employed for crystallization in each case. It should be particularly noted that the values for the ring D α-ketols have been corrected for a loss of approximately 30% during paper chromatography. Such a loss has been found to occur in our laboratory for pure samples of these compounds. Correction was made to compare more directly the figures for the separated ring D α-ketols with those of the other metabolites which were not subjected to paper chromatography. Even this correction, however, did not result in close agreement between the radioactivity found in the ring D α-ketolic fraction from the partition column, which was similar to that of the estrone fraction, and that in the separated ring D α-ketols of Table VI. This discrepancy could either be due to removal of contaminating radioactivity or to the known chemical lability of these compounds. The latter possibility appears less likely since care was taken to avoid vigorous chemical treatment (e.g. high temperature, alkali) at all stages of the work. It can be seen that the ^{3}H in the form of identified urinary metabolites in Table VI amounted to 38–62% of the ^{3}H in the urinary extracts of these experiments (Table I).

Table VII contains similar data on the conversion of ^{14}C-labeled 16-oxygenated compounds to urinary metabolites in four experiments. A survey of

TABLE VII

Conversion (% dose) of ^{14}C-labeled 16-oxygenated estrogens to urinary metabolites as calculated from crystallization data

	Steroid injected						
	16-KE_2-^{14}C		16-epiE_3-^{14}C		E_3-^{14}C		16-epiE_3-^{14}C
Metabolite	G	S	G	S	G	S	G
16β-OHE_1	0.5	—	1.7	—	—	—	—
16-KE_2	5.6	1.1	2.6	—	0.2	—	4.9
16-epiE_3	4.5	0.5	32.8	0.7	0.1	<0.1	21.5
E_3	21.0	2.5	10.4	0.3	42.7	2.7	4.9
Total	31.6	4.1	47.5	1.0	43.0	2.7	31.3

Tables II, III, IV, and V shows that the urinary metabolites of these compounds were generally more radiochemically homogeneous following column or paper chromatography than were the metabolites of estradiol-^{3}H. This is borne out by the finding that of the ^{14}C in the urinary extracts (Table I), 73–100% was identifiable as the metabolites of Table VI.

Discussion

It can easily be seen from the results of this study that of eight urinary metabolites of injected estradiol-^{3}H in the human male, by far the greater portion appears to be conjugated as glucosiduronate, or at least is released by β-glucuronidase treatment. The main component of the fraction released by solvolysis, in all probability representing sulfate conjugates, is estrone. In most instances, small but detectable amounts of the other identified phenolic steroid

metabolites appeared to be conjugated in the latter form. The individual ring D α-ketols, as urinary metabolites of injected estradiol-^{3}H, appear to be considerably less quantitatively significant than was reported earlier from this laboratory (14). However, it should be noted that the latter results were obtained by a much more indirect approach. Also, there remains the possibility that the chemical lability of these compounds may have contributed to losses during separation.

The identified urinary metabolites of injected 16-oxygenated phenolic steroids are obviously almost wholly in the form of glucosiduronates. The pattern of conversion products of 16-ketoestradiol-^{14}C in the present work agrees well with that reported by Levitz *et al.* (7). Both in this experiment and in the one where 16-epiestriol-^{14}C was injected together with estradiol-^{3}H, urinary 16β-hydroxyestrone was significantly labeled with both isotopes whereas 16α-hydroxyestrone, although containing ^{3}H, was practically devoid of ^{14}C. This suggests the latter to be predominantly a hydroxylation product of estrone and the 16β isomer a product, at least partially, of a preformed 16-oxygenated compound. Although the conversion to 16β-hydroxyestrone was not large in either experiment, the possibility that it could have arisen from a contaminant of the administered ^{14}C-labeled steroids appears remote since the purity of the latter was such that only a very high degree of conversion of any hypothetical contaminant could have provided the results obtained. Such efficient metabolic conversions are not generally encountered among the phenolic steroids.

The considerable degree of conversion of 16-epiestriol to 16-ketoestradiol and estriol as compared with that of estriol to 16-ketoestradiol and 16-epiestriol supports an earlier finding in this laboratory (14). Perhaps significant is the finding of an extremely high conversion of injected 16-epiestriol to estriol in a subject who showed a considerable production of the latter compound from administered estradiol. It should be noted that Nocke *et al.* (8) reported the conversion of administered 16β-hydroxyestrone, not only to urinary 16-epiestriol (13.8%) but also to estriol (13.3%) in man. As discussed by these authors, this might relate to the conversion of 16-epiestriol to estriol via 16-ketoestradiol or to the oxidation of 16β-hydroxyestrone to 16-ketoestrone with subsequent reduction to the triols. The part which might be played by 16-ketoestrone in the present work has not been evaluated.

In the experiment involving estriol administration, little, if any, metabolism of this compound occurred as judged by urinary metabolites. However, it should also be noted that in this subject very little of the triols was produced from estradiol as compared with the other experiments. Indeed, more 2-methoxyestrone than estriol was formed from estradiol in this case, a pattern found in hyperthyroidism (18), although no features of this disorder were apparent. Levitz *et al.* (6) were first to show the conversion of estriol to 16-epiestriol via 16-ketoestradiol in man. More recently others have been unable to confirm this finding (19).

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NOTES

FORMATION OF 16-KETO ESTRONE FROM 16-KETOESTRADIOL-17β BY HUMAN UTERINE FIBROID IN VITRO

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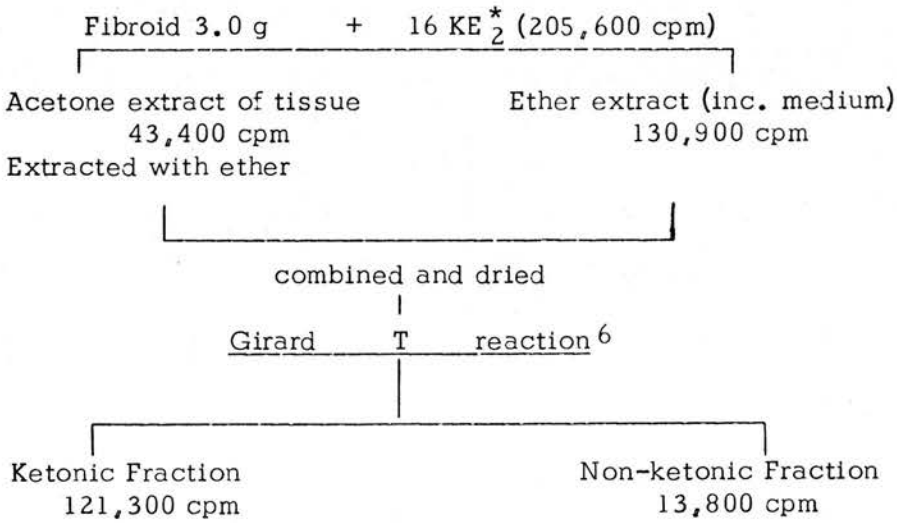
Interconversion of estrone and estradiol-17β by human liver, kidney and breast tissue has been demonstrated in vitro [1]. Breuer [2] has shown conversion of 16-ketoestradiol-17β to the isomeric triols, 16-epiestriol and estriol by slices of human liver. Isolation of C^{14}-labelled 16-ketoestrone from urine following an injection of estrone-16-C^{14} suggests that this metabolite may function as an intermediate in estrogen triol formation [3]. Human tissues convert 16-ketoestrone to 16-ketoestradiol-17β, estriol and 16-epiestriol *in vitro* [2]. According to Szego and Roberts [4] estriol in immature rats induces a more rapid uterine weight increase than does estrone or estradiol-17β.

In human females during the age of reproduction fibroid tumours (leiomyomata) are commonly found in the myometrium. The histological origin of these tumours is still an unsolved problem, nevertheless it is believed that they grow under the influence of circulating estrogens [5].

We wish to report upon the *in vitro* metabolism of estrogens

by human fibroid. Uterine tissue was obtained from a 45-year old female who had a hysterectomy because of paraovarian cyst and leiomyomata (fibroids). The patient was nulliparous with normal menstrual periods, and the surgery was done in the early proliferative phase of the menstrual cycle.

Fibroid tissue was dissected from surrounding uterine muscle, minced, and incubated in Krebs Ringer phosphate buffer + 200 mg % glucose, at pH 7.4 under 0_2 atmosphere at 37.5°C for 3 hrs. The incubation vessel contained 3.0 g tissue, 20 ml incubation medium and 2.9 µg 16-ketoestradiol-17β-16-C^{14} (16 KE_2^*) (205,600 cpm counted at 60% efficiency). Prior to the experiment 16 KE_2^* was purified by paper chromatography. After incubation the aqueous medium and the tissue were extracted according to the following scheme:



The ketonic fraction, to which 25 µg 16-ketoestradiol-17β carrier had been added, was chromatographed on Whatman No. 1

chromatographic paper in the system n-hexane : benzene : 70% methanol (20 : 80 : 100) with a pilot of 16-ketoestradiol-17β on a separate strip. The radioactive zones on the paper were located by means of a 4 π recording and integrating strip Scanner (Atomic Accessories, Inc.). As shown in Fig. 1 radioactive zones less polar than 16-ketoestradiol-17β were present. On repeated chromatography in the system methyl-cyclohexane : toluene : 80% methanol (225 : 275 : 500) the less polar component which had the same mobility as authentic 16-ketoestrone (Rf 0.3), was separated from 16-ketoestradiol (Rf 0.1). This zone was eluted, combined with 230 μg of unlabelled 16-ketoestrone carrier and rechromatographed in the same system. The radioactivity remained

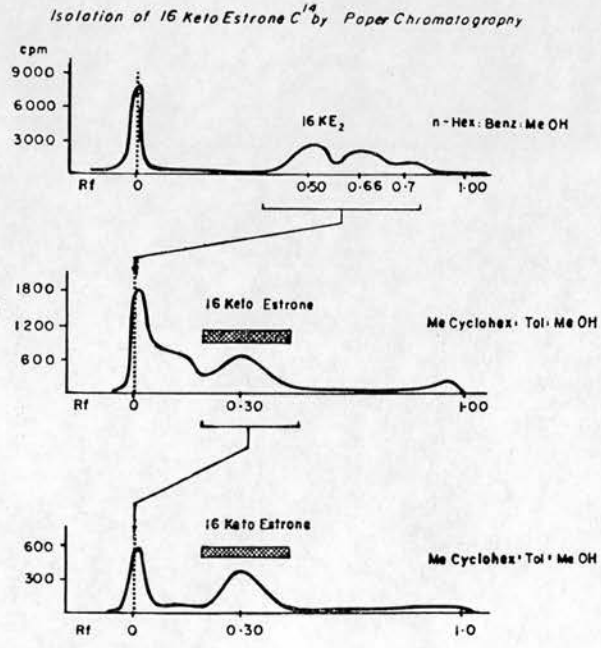


Figure 1. Isolation of 16 Keto Estrone C^{14} by Paper Chromatography.

associated with the 16-ketoestrone carrier. After elution this zone contained 3565 cpm when counted in a Packard Liquid Scintillation spectrometer at 60% efficiency and accuracy $\pm$ 5%. The amount of 16-ketoestrone was determined quantitatively by a modified method based on that of Mitchell and Davies[7]. The isolated metabolite had a specific activity of 4970 cpm/μM. It was reduced by sodium borohydride (10 mg/ml) in 50% ethanol at room temperature for 18 hrs. The reduced material was extracted with peroxide-free ether and chromatographed on Whatman No. 1 paper in the system n-hexane : benzene : 70% methanol (20:80:100). Two polar zones with the chromatographic mobility of estriol (Rf 0.05) and 16-epiestriol (Rf 0.32) were isolated. The specific activity of the estriol was 5090 cpm/μM and of the 16-epiestriol 4980 cpm/μM which indicates that both the radioactivity and the weight were derived from the parent substance.

Radioactive 16-ketoestradiol-17β-C^{14} when incubated without the tissue and subjected to the same procedure of separation did not yield any radioactive product which would correspond to 16-ketoestrone. This strongly suggests that the 16-ketoestrone was formed by the fibroid tissue. Chromatographic separation of the non-ketonic fraction from the tissue incubate yielded two radioactive zones which had the chromatographic characteristics of estriol and 16-epiestriol using paper and thin layer chromatography[8]. These zones did not appear when the 16 KE_2^* was incubated without the tissue.

The recovery of unaltered 16 KE_2^* after incubation with tissue

followed by extraction, chemical and chromatographic separation was 11% whereas in the absence of the tissue it was of the order of 78%, thus showing that 16-ketoestradiol-17β is rapidly metabolized by the fibroid tissue. The ketonic fraction from the tissue incubate was composed of 60% of a highly polar zone, 18% of unaltered 16 KE_2^* and 3% of 16-ketoestrone. In the non-ketonic fraction substances with the chromatographic characteristics of estriol and 16-epiestriol accounted respectively for 16% and 11% of the radioactivity, the remainder was less polar.

These findings indicate that uterine fibroid tissue in vitro can carry out reduction at C_{16} and also oxidation at C_{17} of the estrogen molecule.

Reduction at C_{17} of estrone 16-C^{14} and formation of labelled estradiol-17β has been observed in our laboratory with human endometrium, Fallopian tube and fibroid tissue. The conversion occurs to an extent of 1 to 2% per gram of tissue [9]. Apparently normal myometrium so far has shown little activity in converting estrone to estradiol-17β. Formation of estrogen triols from labelled estrone by these tissues has not been obtained as yet.

The following trivial names are used in the text:

16-ketoestradiol-17β-16-C^{14} = [16-^{14}C] 3, 17β-dihydroxy-1, 3, 5 (10)-estratriene-16-one

Estrone = 3-hydroxy-1, 3, 5 (10)-estratriene-17-one

16-ketoestrone = 3-hydroxy-1, 3, 5 (10)-estratriene-16, 17-dione

16-ketoestradiol-17β = 3, 17β-dihydroxy-1, 3, 5 (10)-estratriene-16-one

Estradiol-17β = 3, 17β-dihydroxy-1, 3, 5 (10)-estratriene

Estriol = 3, 16α, 17β-trihydroxy-1, 3, 5 (10)-estratriene

16-epiestriol = 3, 16β, 17β-trihydroxy-1, 3, 5 (10)-estratriene

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FORMATION OF LABELLED 2-METHOXYESTRONE BY RAT LIVER SUBCELLULAR FRACTIONS

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Abstract

This communication deals with the biosynthesis of 2-methoxyestrone from estrone and 2-hydroxyestrone. Cell free preparations (8000 g supernatant) from livers of immature female Wistar rats contain enzyme systems which activate methionine, orthohydroxylate estrone, transfer the methyl group from methionine to position 2 of 2-hydroxyestrone and reduce the 17-keto group. The orthomethyl-transferase activity may be partially blocked by large amounts of 2-hydroxyestrone. Ortho methylation of estrone is inhibited by estriol, epinephrine, 3, 4-dihydroxy benzoic acid and chlorogenic acid.

The isolation and identification of 2-methoxyestrone as a natural urinary metabolite of labelled estradiol-17β in human subjects was first achieved by Kraychy and Gallagher.[1] Soon afterwards, Loke and Marrian isolated this metabolite from the urine of pregnant subjects.[2] Studies by Hobkirk and Nilsen reveal that the urinary excretion of 2-methoxyestrone by pregnant humans seldom follows the pattern of other estrogen metabolites and shows a marked variation among individuals.[3] Recently Breuer[4] showed that 2-methoxyestradiol-17β and 2-methoxyestriol also appear in human urine. Methylation of 2-hydroxyestradiol-17β to 2-methoxy-

estradiol-17β has been demonstrated "in vitro" by liver tissues from rat, rabbit and human.[5] As shown by King[6], rat liver preparations in vitro transform estriol to 2-hydroxyestriol and 2-methoxyestriol.

We wish to report on the formation of 2-methoxyestrone from estrone and 2-hydroxyestrone by rat liver preparations in vitro.

Materials and Methods:

Tissues for incubation were obtained from immature female Wistar rats weighing 50±5 grams. The animals were sacrificed by a blow on the head and the liver was removed immediately. A 10% (w/v) homogenate was prepared in 0.25 M sucrose and the subcellular fractions were separated by means of a refrigerated Beckman Spinco Type (Model L) Ultracentrifuge. The nuclei and cell debris were sedimented at 700 times gravity and the mitochondria at 8000 times gravity for 30 minutes. The supernatant obtained after centrifugation at 8000 times gravity is referred to in the text as the "microsomal" preparation; it contains microsomes + soluble fraction. For incubation 5 ml of disrupted cell preparation were diluted with 5 ml of 0.2M phosphate buffer pH 7.4 containing 1.5 μM methionine methyl-^{14}C (sp.act.6.31x10^{6}cpm/μM)* as a methyl donor, estrone and co-factors ** 2.3 μM NAD, 2μM NADP, 15 μM glucose-6-phosphate, 10.0 μM adenoisine triphosphate and 279 μM magnesium chloride. For some experiments estrone-16-^{14}C and non-

labelled l-methionine were used. This mixture was incubated for 2 hours under oxygen atmosphere at 37.5°C in a Dubnoff Metabolic Incubator. Immediately after incubation the preparation was extracted with peroxide-free ether. The extract was evaporated to dryness and subjected to a Girard T separation[7] for 18 hours at room temperature. After this reaction the non-ketonic fraction was extracted with peroxide free ether. The aqueous residue containing the ketonic Girard complex was hydrolyzed with sulfuric acid and the liberated ketonic material was extracted again with ether. Ether extracts were evaporated to dryness and chromatographed. The radioactivity of extracts and eluates was measured in a Packard Liquid Scintillation spectrometer with an efficiency for carbon-14 of 60%. Radioactive zones on paper chromatograms were detected by means of an integrating and recording 4π windowless gas flow strip scanner (Atomic Accessories Inc.). In some instances radioautography was also used.

Isolation of 2-Methoxyestrone

The ketonic fractions from the incubation media, when chromatographed on Whatman No. 1 filter paper in the propylene glycol: methylcyclohexane system, separated into several radioactive zones. The zone having the mobility of 2-methoxyestrone (2 MeE_1) appeared only when estrone or 2-hydroxyestrone (2-OHE_1) was present in the incubation medium (Figure 1). On further chroma-

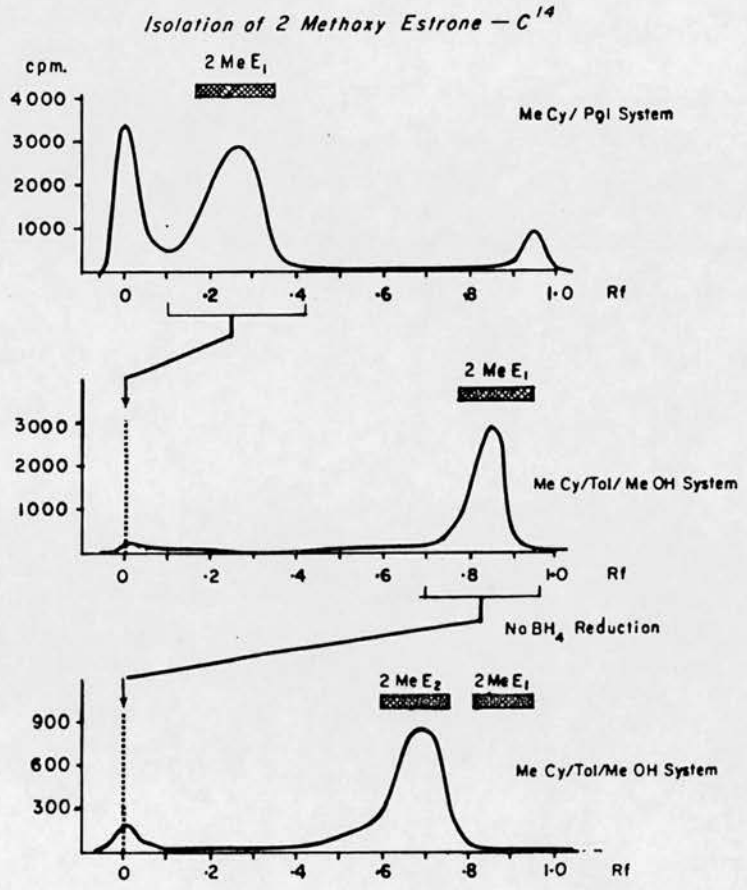

Figure 1: Isolation of 2 methoxyestrone-^{14}C. Scanning patterns of chromatograms. 2 MeE_1 and 2 MeE_2 = areas of authentic pilots.

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tography in the methylcyclohexane : toluene : 80% aqueous methanol system (MeCy:Tol:MeOH) (225:275:500) this zone remained homogeneous and the radioactivity did not separate from the authentic 2-methoxyestrone carrier. After elution and subsequent chemical reduction with sodium borohydride (10 mg/ml in 50% ethanol for 18 hours at room temperature) both the carrier and the radioactivity remained together and behaved like 2-methoxyestradiol-17β (2MeE_2).

Pooled 2-methoxyestrone methyl-^{14}C was chromatographed on a celite column using the solvent system n-hexane: benzene: 70% methanol (98:2:100)[7]. The isolated 2-methoxyestrone fraction when chromatographed on Silica Gel G (Stahl) thin layer system "A"[8] had the same mobility as authentic 2-methoxyestrone. A portion of 2-methoxyestrone methyl-^{14}C was diluted with authentic non-labelled 2-methoxyestrone to a specific activity of 44, 200 cpm/μM and acetylated with acetic anhydride in pyridine for 18 hours at room temperature. The acetylated material was chromatographed in the propylene glycol: methylcyclohexane system and it showed only one radioactive zone of R_f 0.39 (2-methoxyestrone pilot R_f 0.14). A part of the chromatogram when tested with dilute (1: 10) Folin reagent for free phenol group showed only a slowly developing trace of blue color in the 2-methoxyestrone acetate region, and no color in the 2-methoxyestrone region. Free 2-methoxyestrone in comparable amount under the same conditions with Folin reagent rapidly yields a dark blue color. The specific activity of the eluted 2-methoxyestrone methyl-^{14}C acetate zone was 44,100 cpm/μM, thus showing no change in specific activity following acetylation. The weight of 2-methoxyestrone and its acetate was determined by the Ittrich modification of the Kober reaction[9].

Factors affecting 2-methoxyestrone formation:

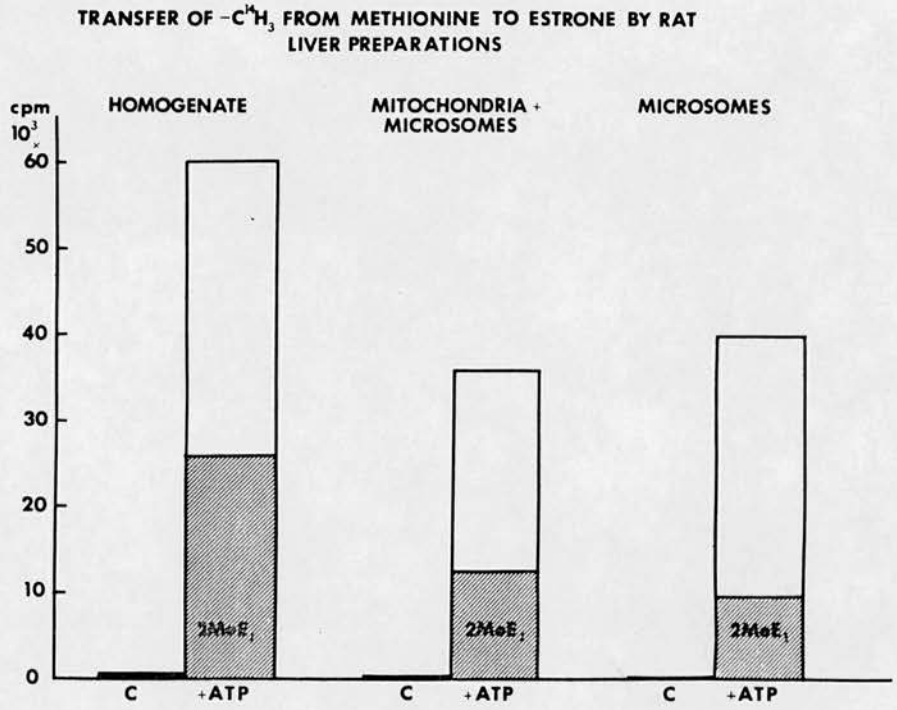

Figure 2: Formation of 2 methoxyestrone-methyl-^{14}C by cell-free preparations.

C - no ATP
+ ATP 10 μM

Total bar - radioactivity in ketonic fraction.
Shaded bar - radioactivity in 2-methoxyestrone zone.

The dependence of methoxylation on exogenous ATP and the location of the enzyme systems involved has been studied. Cell-free rat liver preparations failed to incorporate radioactivity from methionine methyl-^{14}C into the ketonic fraction in the absence of ATP. The whole homogenate was most efficient in methoxylation but the microsomal fraction (8000 g supernatant) contained the necessary enzymes for these reactions. (Figure 2). In the

presence of ATP 2-methoxyestrone was formed and it accounted for less than one half of the total radioactivity present in the ketonic fraction. The remainder was more polar material which has not been identified.

The formation of 2-methoxyestrone from estrone and 2-hydroxyestrone by rat liver "microsomal preparation" has been investigated. The concentrations of all the co-factors and methionine methyl-^{14}C were identical in the samples. As the amount of estrogen increased, the radioactivity in the ketonic fraction and in the 2-methoxyestrone zone increased (Figure 3); 2-hydroxyestrone led to a much greater formation of 2-methoxy-estrone than did estrone as a substrate. The non-ketonic fractions showed an increase in radioactivity as the amounts of estrone or 2-hydroxy-estrone were increased in the incubation mixture (Table 1). On chromatography in the MeCy:Tol:MeOH system this fraction yielded a radioactive zone which had the same mobility as 2-methoxyestradiol-17β, and two other zones, one retained on the starting line and the other less polar than $2MeE_2$. On thin layer chromatography the $2MeE_2$ zone behaved like authentic 2-methoxyestradiol-17β. It is of interest to note that 2-hydroxyestrone yielded proportionately more radioactivity in the non-ketonic fraction and in the $2MeE_2$ zone than did estrone. It is unlikely that the 2-hydroxyestrone used for incubation could contain 2-hydroxyestradiol-17β since on chromatography this

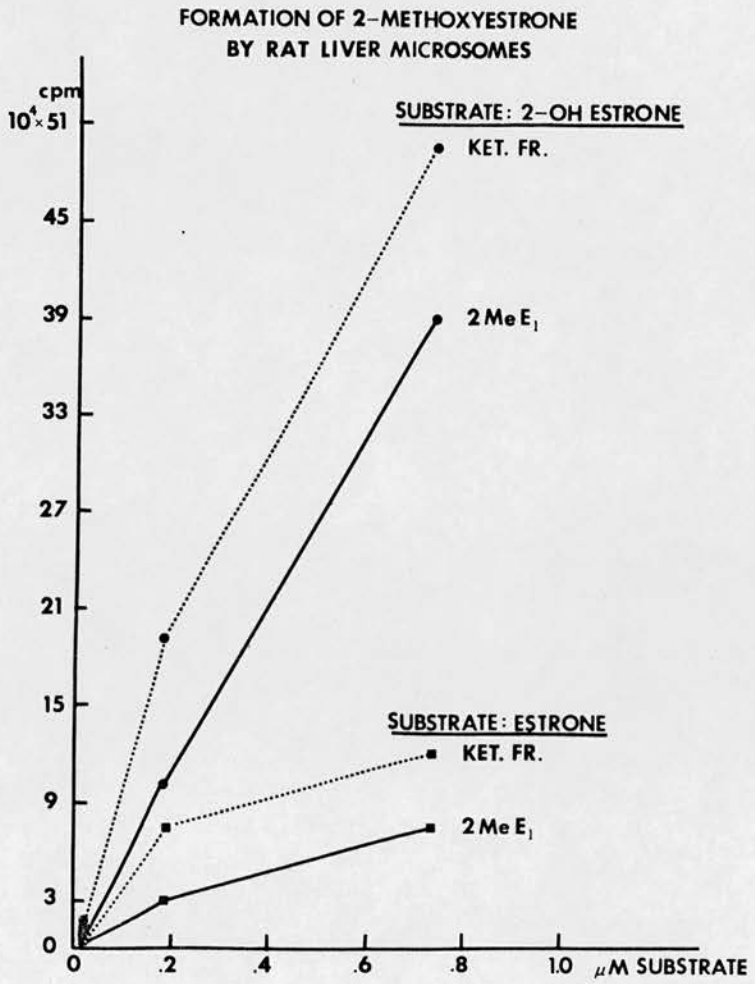

Figure 3: Formation of 2 methoxyestrone by rat liver "microsomal" preparation (8000 g supernatant).

Dotted line - radioactivity in the ketonic fraction.
Solid line - radioactivity in 2 methoxyestrone zone.

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steroid did not separate or yield any compound which would correspond to 2-hydroxyestradiol-17β. Also, estrone used for incubation was free from other contaminants. These results suggest that the rat liver "microsomal" preparation carries out "in vitro" activation of methionine besides ortho methylation in

Table 1

Formation of 2-methoxy derivatives from estrone and 2-hydroxyestrone by rat liver 8000g supernatant in the presence of methionine methyl - ^{14}C

Substrate	µM	Ketonic fraction cpm	2 MeE_1 cpm	Non-ketonic fraction cpm	2 MeE_2 cpm
Estrone	0.018	10,560	2,400	6,900	800
Estrone	0.180	76,800	28,700	25,700	6,100
Estrone	0.740	133,300	72,000	37,600	3,400
2-0H-E_1	0.024	18,500	3,400	9,500	1,200
2-0H-E_1	0.170	193,300	102,000	89,300	12,700
2-0H-E_1	0.730	491,800	392,000	222,500	142,000

2-0H-E_1 = 2-hydroxyestrone
2 MeE_1 = 2-methoxyestrone
2 MeE_2 = 2-methoxyestradiol-17β

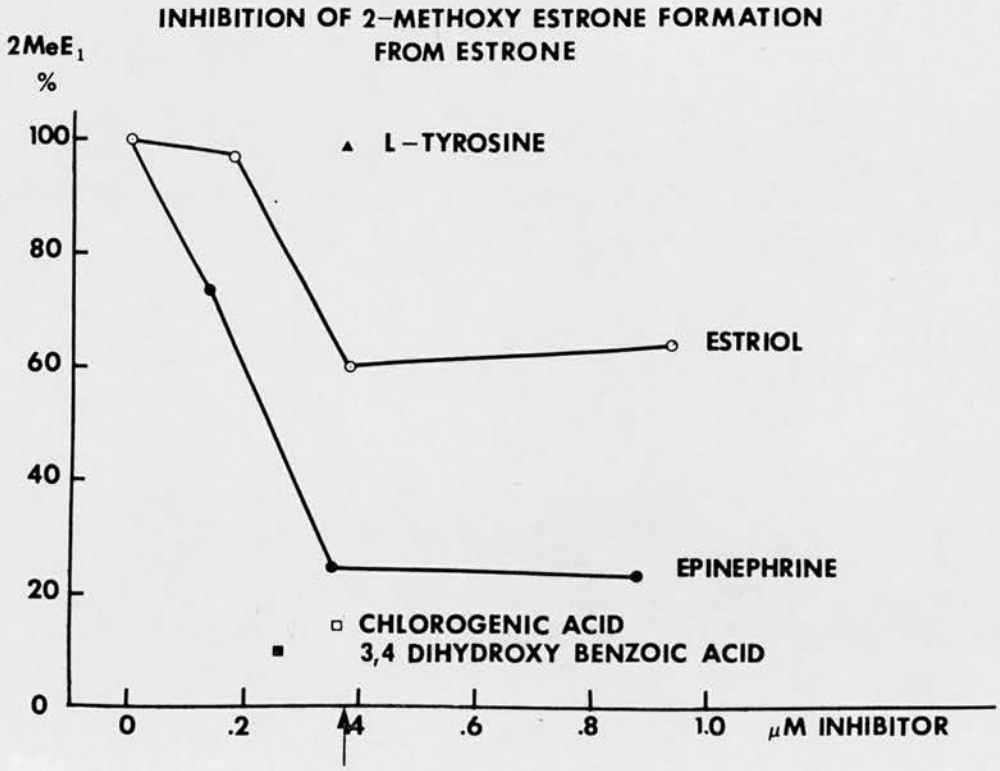

Figure 4: Inhibition of 2-methoxyestrone formation from estrone and methionine-methyl-^{14}C by rat liver "microsomal" preparations (8000 g supernatant).

Initial concentration of estrone = 0.37 μM, indicated by arrow.

Amount of 2-methoxyestrone formed in absence of inhibitor = 100%

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position 2 of ring A and reduction of the 17 keto group.

The effect of tyrosine, estriol and various catechols on orthomethylation has been investigated. Rat liver "microsomal" preparations were incubated with 0.37 μM of estrone in the presence of methionine methyl-^{14}C and co-factors as outlined earlier (Figure 4). Tyrosine had no effect on $2MeE_1$ formation.

Increasing doses of estriol decreased the formation of $2MeE_1$-^{14}C only to a certain degree. Epinephrine produced a more pronounced inhibitory effect with the same pattern. Plant catechols, such as 3, 4 dihydroxy benzoic acid and chlorogenic acid, markedly inhibited estrone conversion to 2-MeE_1-^{14}C.

Incubation of rat liver microsomal preparations with estrone and increasing amounts of estriol produced two effects. As the initial concentration of estriol increased the synthesis of 2-methoxyestrone decreased and estriol became metabolized to increasing amounts of non-ketonic material which behaved chromatographically on paper in the hexane : benzene : 70% methanol (20:80:100) system, and on thin layer systems, as 2-methoxyestriol. When estrone was incubated in the absence of estriol, the non-ketonic fraction did not contain any zone which would correspond to 2-methoxyestriol (Figure 5).

It has been suggested by King[6] and also by Breuer[5] that methylation is preceded by 2-hydroxylation. An attempt was therefore made to block ortho-methyl transferase activity with large amounts of 2-hydroxyestrone. In this series of experiments estrone-16-^{14}C and unlabelled methionine were used, the co-factors concentration being the same as in previous experiments. Estrone 16-^{14}C was metabolized to a product which had the same chromatographic characteristics as had 2-methoxyestrone. As the initial concentration of 2-hydroxyestrone in the incubation medium

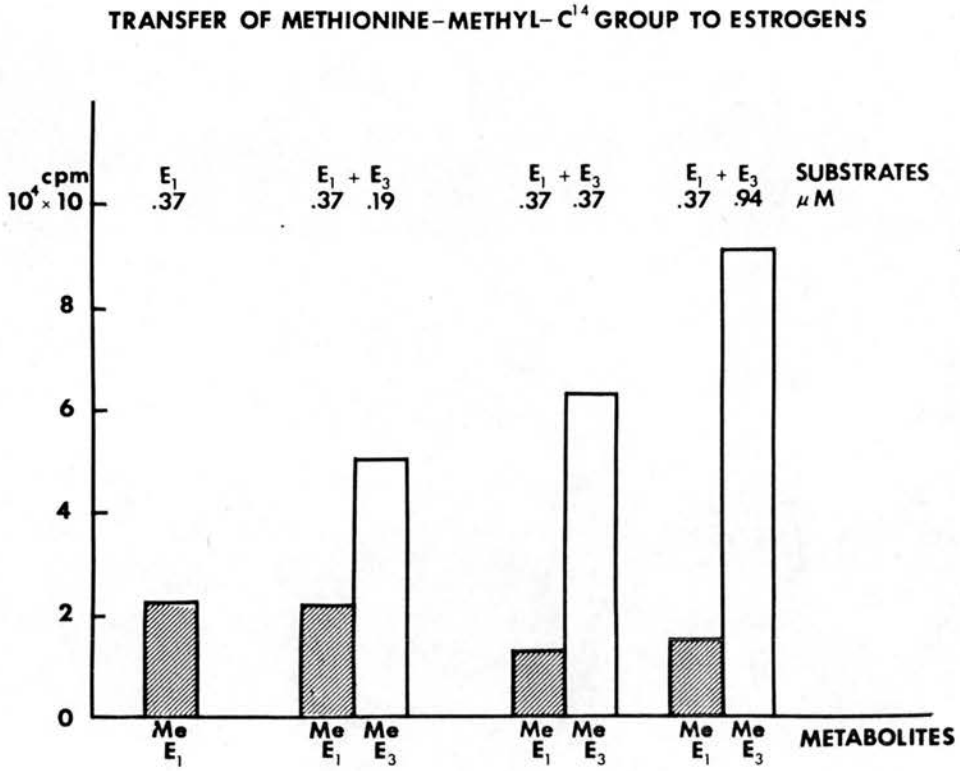

Figure 5: Transfer of methionine methyl-^{14}C group to estrone and estriol by rat liver "microsomal" preparation (8000 g supernatant).

MeE_1 - 2-methoxyestrone
MeE_3 - 2-methoxyestriol

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increased an increment was observed in the radioactivity which had the chromatographic mobility of 2-hydroxy estrone in the MCy:Tol: MeOH paper chromatographic system and in system "A" for thin layer chromatography (Table 2). The chemical instability of this compound made further characterization of the 2-hydroxyestrone zone difficult.

Discussion:

Ortho methylation of estrone by rat liver subcellular fractions

Table 2

Formation of labelled 2-hydroxyestrone from estrone-16-^{14}C

Steroids added		Isolated product	
Estrone-^{14}C	2-OH-estrone unlabelled	2-OH-Estrone-^{14}C fraction	cpm
0.37 μM	None		8,940
0.37 μM	0.146 μM		13,960
0.37 μM	0.39 μM		19,210
0.37 μM	0.92 μM		20,450

Estrone-16-^{14}C 0.37 μM = 982,700 cpm

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in the presence of methionine involves a series of biochemical reactions, which includes activation of methionine, ortho-hydroxylation of estrone and the transfer of the methyl group from S-adenosyl-l-methionine to 2-hydroxyestrone. It has been shown by Cantoni[10,11] that mammalian liver tissue contains a methionine-activating enzyme. In the activation reaction adenosine triphosphate (ATP) in the presence of magnesium ions reacts with methionine forming S-adenosyl-l-methionine (SAMe) and releases phosphate and pyrophosphate. It is well known that SAMe functions as a methyl donor in a variety of biochemical methylation reactions. In their studies of catecholamine metabolism Axelrod and Tomchick[12] isolated from the soluble

fraction of rat liver an enzyme, ortho methyl transferase, which catalyzed the transfer of the methyl group from SAMe to position 3 of epinephrine. Recently Breuer et al [5] showed that this enzyme also methylates 2-hydroxyestradiol-17β to 2-methoxyestradiol-17β. In this latter reaction the K_m of 8.7×10^{-4}M is higher than that for epinephrine (K_m 1.2×10^{-4}M) suggesting that 2-OH-E_2 has a lesser affinity for the enzyme. These findings may explain the inhibitory action of epinephrine in 2-methoxyestrone formation by the rat liver subcellular fraction. Inhibition of ortho-methylation by the plant catechols, chlorogenic and 3,4 dihydroxy-benzoic acid, may be explained on similar grounds. Mashri et al[13] have shown that rat liver slices in vitro, when incubated with 3,4 dihydroxy benzoic acid, give rise mainly to 3-methoxy, 4-hydroxy benzoic acid and its glycine conjugate and also to a small amount of 3-hydroxy, 4-methoxy benzoic acid. In the present study no attempts were made to separate epinephrine, 3, 4 dihydroxy benzoic acid or chlorogenic acid metabolites.

Formation of 2-hydroxyestrone from estrone by rat liver subcellular fractions has been shown, althougn definite proof, in the form of the isolation of this intermediate, is still lacking. King[6], however, has demonstrated the formation of 2-hydroxy-estriol from estriol by rat liver microsomes. It is of interest to note that estriol when incubated together with estrone reduced the yield of 2-methoxyestrone and gave rise to 2-methoxyestriol. This

observation may be the result of a more efficient 2-hydroxylation of estriol than of estrone and the availability of a 2-hydroxylated compound which acts as a rate-limiting factor in ortho methylation. The far greater yield of 2-methoxyestrone from 2-hydroxyestrone than from estrone is in favour of this explanation. Ortho-methylation reactions of catechols are rather widespread in mammalian organisms and the activity has been demonstrated in kidney, adrenal, ovary and testis[14]. Tissue mince from human endometrium, Fallopian tube, fibroid tumor, functioning ovary and menopausal ovary activates methionine-methyl-^{14}C without exogenous ATP and forms methoxy estrogens from 2-hydroxyestradiol-17β and 2-hydroxyestrone[15]. Biochemical methylation is one of the mechanisms of inactivation of endogenous and exogenous catechols. In the case of estrogens 2-hydroxylation reduces the biological potency some 100-fold and a subsequent methylation of 2-OH-estrone and 2-OH-estradiol-17β reduces the activity a further 10-fold as shown by Martin[16] using the intravaginal blue tetrazolium assay. In other reports where uterine weight assay has been used, the potency of 2-methoxy estrogens may be even lower. 2-Methoxy and also 2-hydroxy estriol are inactive in the mice vaginal assay.[16] Recently, Mittermayer and Breuer[17] demonstrated that rat liver microsomes catalyze the demethylation of 2-methoxyestradiol-17β to 2-hydroxyestradiol-17β. This reaction, however, occurred to a rather small extent of about 2.5%. In vivo administration of 2-methoxyestrone

to human subjects is apparently followed by demethylation with the excretion of 2-hydroxyestrone[18]. Brown[19] has demonstrated in vivo the demethylation of estrone, estradiol 17β and estriol 3-methylethers. In terms of biological potency demethylation may represent an activation mechanism. The nature of the demethylating enzyme system and its tissue distribution is still not known.

The following trivial names and abbreviations are used in the text:

Estrone = 3-hydroxy - 1,3,5(10) - estratriene -17- one.
(E_1)

Estradiol 17β = 3, 17β -dihydroxy - 1,3,5(10) estratriene.
(E_2)

Estriol = 3, 16α, 17β -trihydroxy - 1,3,5(10) estratriene.
(E_3)

2 Hydroxyestradiol 17β = 2,3,17β -trihydroxy- 1,3,5(10) - estratriene.
(2-0H-E_2)

2 Hydroxyestrone = 2,3, -dihydroxy - 1, 3, 5(10) - estratriene -17- one.
(2-0H-E_1)

2 Methoxyestrone = 2-methoxy, -3-hydroxy- 1,3,5(10) - estratriene -17- one.
(2-MeE_1)

2 Methoxyestradiol 17β = 2 methoxy-3,17β -dihydroxy - 1,3,5(10)-estratriene.
(2-MeE_2)

2 Methoxyestriol = 2-methoxy-3,16α, 17β -trihydroxy - 1,3,5(10) -estratriene.
(2-MeE_3)

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* New England Nuclear Corp. NEC -165

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METABOLISM OF DOUBLY LABELLED ETHYNYLESTRADIOL-3-CYCLOPENTYL ETHER IN WOMEN[1]

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ABSTRACT

The metabolic fate of orally administered 17α-ethynylestradiol-3-cyclopentyl ether (EECPE) labelled with ^{3}H in the steroid nucleus and with ^{14}C in the cyclopentyl group has been studied in women in two independent laboratories. Radioactivity was excreted over a period of up to 127 days, and unchanged EECPE was recovered from fat at 2 and 3 days after ingestion. Substantial quantities of ethynylestradiol were identified in the processed urine by recrystallization with carrier. Three other urinary metabolites were detected; the least polar contained ^{3}H and ^{14}C but was not EECPE. This metabolite is probably present in the urine over the entire excretion period. The most polar metabolite contained only ^{3}H and was quantitatively prominent in urines from the first few days collections but disappeared from the excretion pattern with time. A metabolite of intermediate polarity increased in relative amount during the collection period.

INTRODUCTION

Etherification at position 3 has been reported (4,5) to increase the oral and decrease the subcutaneous activity of ethynyl estradiol (EE). Meli and co-workers (5) have shown that storage in

and release from body fat is the mechanism responsible for this increased oral acitivity, in rats, of ethynylestradiol-3-cyclopentyl ether EECPE as compared with EE. These investigators (6) have also shown that the estrogenic material stored in rat perirenal fat is unaltered EECPE . The results of Epstein et al. (7) have indicated that EECPE has a prolonged estrogenic effect in humans. The present report is the result of two independent studies undertaken to provide information on the storage, rate of excretion and metabolism of radioactive EECPE in human subjects in vivo. Where the experimental procedures differed between laboratories both are given.

Experimental procedure A was carried out at The Worcester Foundation for Experimental Biology and experimental procedure B was carried out at The Montreal General Hospital.

EXPERIMENTAL PROCEDURE

A. Worcester Laboratory

The subjects were two physically normal women who were hospitalized for mental disease. Subject A.P. was 35 years old and weighed 174 lbs., while subject J.A. was 29 years old and weighed 156 lbs. Labelled EECPE was obtained from Warner-Lambert Research Institute, Morris Plains, New Jersey. Each subject received 995 µg of EECPE, absorbed on 215 mg of USP lactose and containing 25 µc of ^{14}C and 250 µc of ^{3}H. The tritium was at positions 6 and 7 of the steroid nucleus while the ^{14}C was at position 1' of the cyclopentyl ring. The material was administered orally.

Daily urine collections were made for the first 6 days after administration, and 24-hour samples were obtained at intervals during the next 127 days and assayed for radioactivity in a Packard Tricarb Liquid Scintillation spectrometer. Appropriate corrections for quenching and for the contribution of one isotope to the assay of the other were made as previously described (8).

Several of the 24-hour urine samples were extracted with chloroform to give the "free" fraction of the excreted radioactivity. The urine was then incubated with Ketodase for 24 hours as described by Flood, et al. (8) and extracted with chloroform.

The incubation was repeated for a further 24-hour period and the chloroform extraction was again carried out. In the case of the first 24-hour samples the residual urine was extracted with ethyl acetate at pH 5.0 then after saturation with sodium chloride at pH 1 (9). A final extraction with butanol was then carried out.

The radioactive metabolites in the glucuronoside fraction of the urine samples were initially separated by countercurrent distribution in the system ethyl acetate-cyclohexane-ethanol-water 1:1:1:1 as described by Gallagher, et al.(10). Preliminary identification of ethynylestradiol was made by thin layer chromatography on silica gel and then confirmed by recyrstallization to constant specific activity with the authentic compound.

B. Montreal Laboratory

Three female subjects (A.C., C.L. and E.B.), aged 27, 53 and 39 years, respectively, were investigated. All three suffered from cancer of the cervix but none was terminal during the study. Each was given 190 µg of the labelled EECPE described in Experimental Procedure A, in a single oral dose. The radioactive dose in each case was 50 µc of ^{3}H and 5 µc of ^{14}C. Complete 24-hour urines were collected daily from subjects A.C. and C.L. for 53 and 25 days, respectively. Subject A.C. underwent a hysterectomy two days after administration of the dose and at this time various tissues, including fat, were obtained. Subject E.B. underwent a similar operation three days following EECPE administration. In this case only fat was obtained.

All urines were assayed for radioactivity using a Nuclear-Chicago model 6725 dual channel liquid scintillation spectrometer. All samples were corrected for quenching and the ^{3}H and ^{14}C counts calculated by a standard method. Various individual urines were extracted with ether following incubation with bacterial β-glucuronidase at pH 6.5 (11). The residual urine was extracted at the same pH with ethyl acetate and then again, with the same solvent, at pH 1, following saturation with NaCl. Finally, butanol extraction was performed. The various extracts were assayed for radioactivity and the ether extract obtained following β-glucuronidase hydrolysis was subjected to partition chromatography on a Celite column with a stationary phase of 80% methanol. Elution was carried out by the stepwise addition of increasing concentrations of benzene in n-hexane.

Tissues other than fat were homogenized in acetone and radioactivity was assayed. Fat was homogenized with acetone, the filtered extract evaporated and the resulting material partitioned between n-hexane and methanol. The methanol extracts were chromatographed as above.

RESULTS

The excretion pattern of both ^{3}H and ^{14}C from the subjects A.P. and J.A. is shown in Fig. 1, expressed as percentage of the administered dose present in each 24-hour urine sample. During the first 6 days, when continuous collections were carried out,

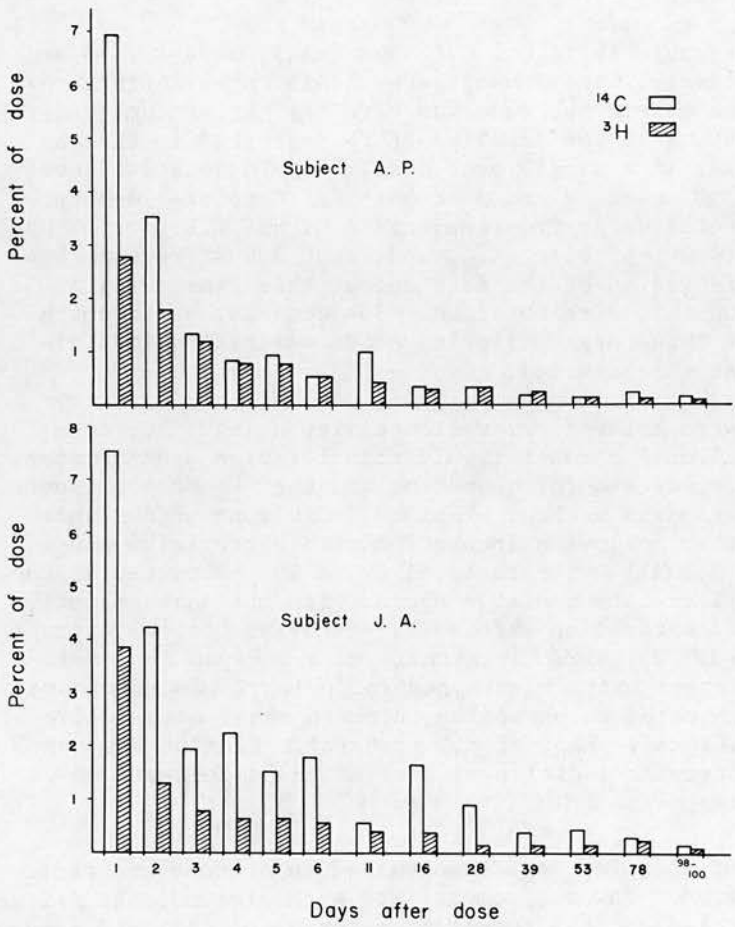

Fig. 1. Urinary Excretion of Labelled Quinestrol.

subject A.P. excreted 14 percent of the ^{14}C dose and 8 percent of the ^{3}H dose, while subject J.A. excreted 19 percent of the ^{14}C and 8 percent of the ^{3}H. For the following 100 days there was a continuous slow excretion of both isotopes. At day 127, very small amounts of radioactivity were still being excreted by A.P., while no radioactivity was detectable in the urine of J.A.

Essentially the same pattern was obtained for subject A.C. (Fig. 2) over 53 days of continuous collection. Similar results were obtained with subject C.L. over 25 days.

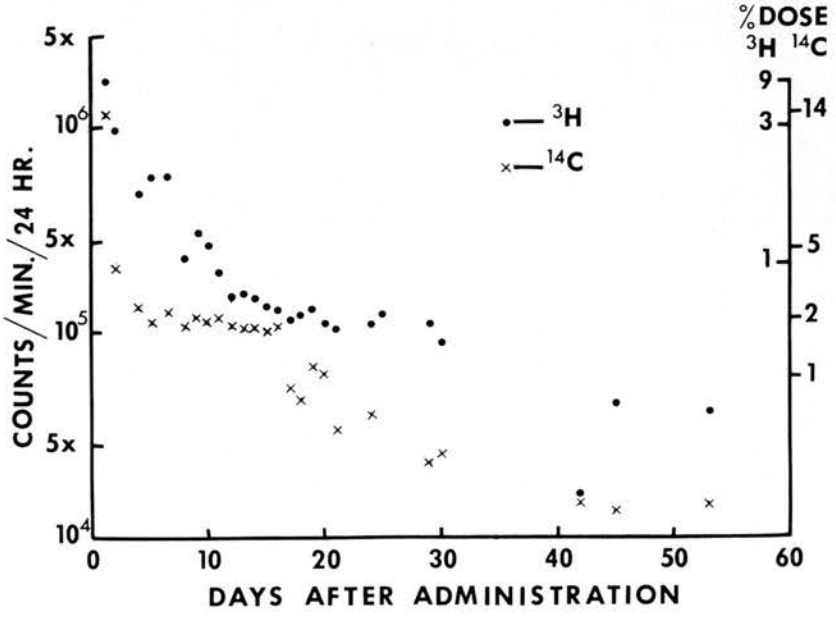

Fig. 2. Urinary excretion of total radioactivity in subject A.C. Recovery after 53 days: ^{3}H = 35%, ^{14}C = 48%.

Table 1. Recovery of radioactivity from the first 24-hour sample of urine from women after administration of double labelled ethynylestradiol-3-cyclopentyl ether. Results are percentages of the total radioactivity in the urine sample.

Day	Subject	Extraction Procedure									
		$CHCl_3$ at neutral pH		$CHCl_3$ after Ketodase		EtOAc at pH 5.0		EtOAc at pH 1.0		Butanol at pH 1.0	
		^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
1	A.P.	0.7	3.9	4.2	56.7	0.6	4.9	28.7	21.3	32.0	9.1
	J.A.	0.7	4.7	4.3	47.8	0.5	5.5	22.6	15.2	22.0	7.1
	A.C.			3.4	32.1[a]	2.3	9.9[b]	13.9	26.1	29.0	14.0
28	A.P.	1.1	2.2	7.9	48.5						
	J.A.	0.2	2.3	2.8	61.1						
53	A.P.	1.5	1.8	14.9	53.5	1.4	4.2				
	J.A.	0.5	1.6	5.7	50.4	0.2	1.9				
98-100[c]	A.P. and J.A.	0.7	0.8	9.2	60.9						

[a]Enzyme=bacterial β-glucuronidase, solvent=ether.

[b]pH used was 6.5.

[c]Represents the pooled urine of both subjects for three days.

Table 1 shows the percentage of the urinary radioactivity from A.P. and J.A. which was extracted from the individual urines of days 1, 28, 53 and the combined urines of days 98, 99 and 100. Only small amounts of either ^{14}C or ^{3}H were extracted from the urine by chloroform prior to hydrolysis. A considerable amount of ^{3}H but very little ^{14}C was extracted after treatment with β-glucuronidase (Ketodase, Warner-Chilcott). Most of the ^{14}C was in a highly polar form and was only extracted by ethyl acetate or butanol at pH 1.

Also included in Table 1 are the results for the extraction of radioactivity from the first day's urine of subject A.C. As above, the majority of the ^{14}C was in a highly polar form resulting from rupture of the ether linkage of the EECPE and further degradation of the cyclopentyl fragment. The figures for A.C. were qualitatively similar to those for A.P. and J.A. although a slightly different extraction scheme was followed. The quantitative differences may be due in part to less complete hydrolysis by the bacterial glucuronidase than by Ketodase and to the greater solubility of the metabolites in chloroform as compared to ether. Only the chloroform and the ether extracts following enzymatic hydrolysis were further examined.

The countercurrent distribution pattern of the ^{3}H in the glucuronoside fractions of the urines from days 1, 53 and 98-100 are shown in Fig. 3 for J.A. and A.P. Three major peaks of radioactivity, A, B and C, are evident. Peak A disappears from the distribution pattern with time, while the relative size of peak B

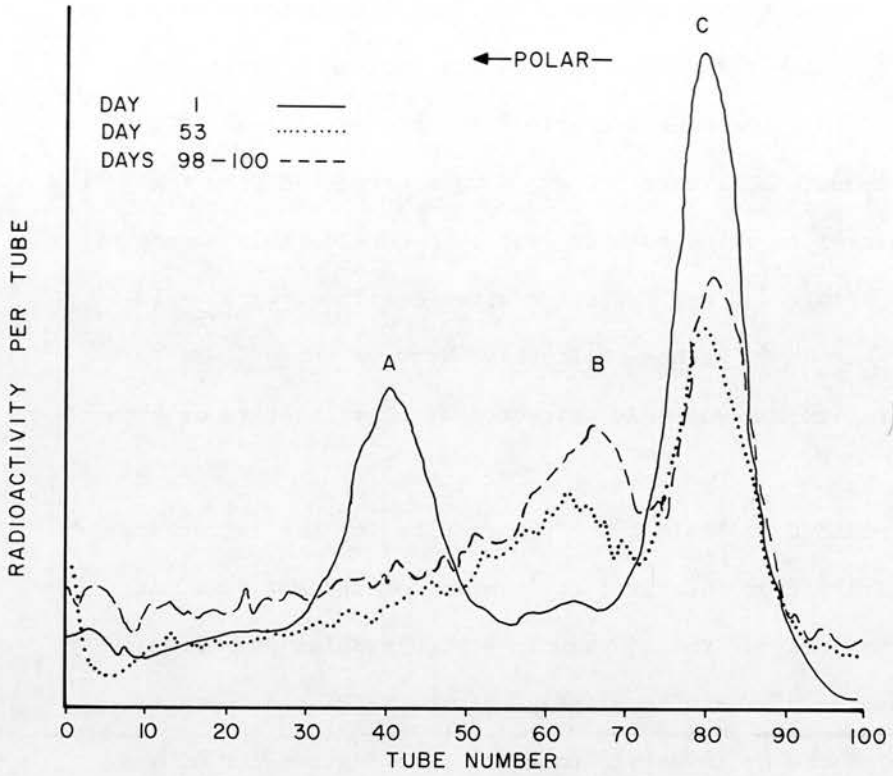

Fig. 3. Countercurrent distribution of the tritium extracted with chloroform, after Ketodase treatment, from the urine of women receiving doubly labelled ethynylestradiol-3-cyclopentyl ether. The system was ethyl acetate-cyclohexane-ethanol-water 1:1:1:1.

to peak C increases. Peak C was found to consist almost entirely of a material containing ^{3}H but no ^{14}C which was similar to ethynylestradiol on thin layer chromatography. An aliquot of peak C from the distributions for each of days 1, 53 and 98-100 was added to authentic ethynylestradiol. Constant specific activity was obtained on recrystallization in each case, at 76, 58, and 84 percent of that calculated from the radioactivity of peak C for the respective days (Table 2.).

Table 2.

Crystallization of radioactivity from Peak C, fig. 3, and Peak 2, fig. 4, with carrier ethynylestradiol

	mg carrier	d.p.m. used (^{3}H)	d.p.m./mg at constant s.a.	% of Peak as E.E.
Day 1	103	2.93×10^5	2200	78
Day 53	65	1.7×10^5	1500	58
Days 98-100	66.5	1.65×10^5	2200	84
Peak 2, fig.4	47.6	1.66×10^5	3000	85

When a portion of the glucuronoside extract of the day 1 urines of J.A. and A.P. was distributed in the countercurrent system carbon tetrachloride-methanol-water 10:7:3, a relatively small peak of radioactivity, less polar than ethynylestradiol, containing both ^{3}H and ^{14}C was detected. On thin layer chromatography this material was not identical with EECPE. This peak appeared to be present in similar extracts from days 98-100, however, the absolute amount was too small for further study. This material is probably that described below as peak 1 from day 1 of subject C.L. and day 15 of subject A.C.

All four radioactive peaks detected on countercurrent distribution were examined on thin layer silica gel plates containing silver nitrate, prepared and used as described by Ercoli, et al. (12). In each case the radioactivity remained at the origin in various ethylacetate-cyclohexane systems, indicating that the materials contained an unaltered 17α-ethynyl group.

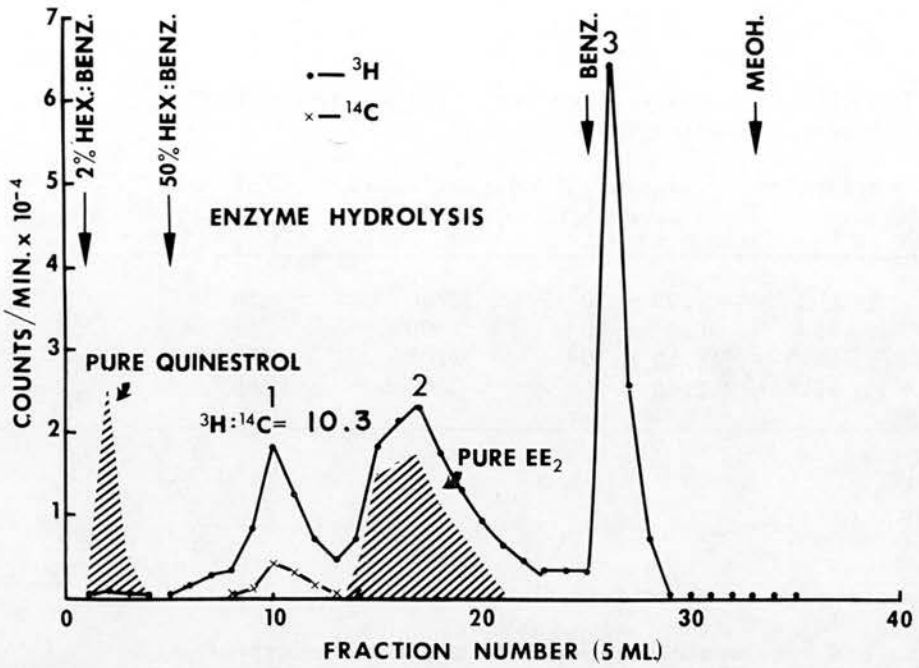

Fig. 4. Celite partition of ether extract of day 1, subject C.L.; 15 x 1 cm. column; recovery from column, ^3H = 98.7%, ^{14}C = 85%. dpm ^3H: dpm ^{14}C of Peak 1 = 10.3

Figure 4 shows the chromatographic separation achieved on the ether-soluble radioactivity following β-glucuronidase hydrolysis of day 1, subject C.L. Three peaks can be seen, one of which, peak 2, containing only ^3H, was shown, by crystallization with unlabelled standard to be composed at least 85% of ethynylestradiol (Table 2.). Peak 1 contained both ^3H and ^{14}C and was more polar than EECPE. Peak 3 contained only ^3H. Approximately 17% of the ^3H activity in the ether extract was present in peak 1, 40% in peak 2 and 34% in peak 3. The pattern obtained by similar treatment of an extract of day 15 from subject A.C. was similar. In this case peak 1 accounted for 23%, peak 2 for 48% and peak 3 for only 13% of the total extract.

The countercurrent pattern (Fig. 3) shows the disappearance of peak A and the appearance of peak B with time. Both of these peaks were more polar than EE and probably are both included in peak 3 of the chromatographic workup.

In subject A.C. no radioactivity could be detected two days after EECPE administration in ovary, uterine wall, endometrium, tube or whole blood. However, a methanol extract of fat (abdominal wall + omentum) contained 11100 d.p.m. of ^{3}H and 1240 d.p.m. of ^{14}C. This material, when chromatographed, showed 92% of the ^{3}H and 80% of the ^{14}C to be eluted in the position shown to be occupied by pure EECPE in Fig. 2. The $^{3}H/\ ^{14}C$ ratio of this material was 10.3. Considerably more fat was obtained from subject E.B., and a methanol extract contained 110,000 d.p.m. of ^{3}H and 10,400 d.p.m. of ^{14}C. Again, all of this activity, when chromatographed, was eluted at the polarity of EECPE with a $^{3}H/^{14}C$ ratio of 10.3. Because of the lipophilic nature of the radioactive material in question, considerable partition between hexane and methanol was performed in order to remove as much contaminating fatty material as possible. This resulted in considerable loss of radioactivity so that a quantitative value for EECPE in the fat was not obtained. Neither was it possible to prove that all of the radioactivity in the fat was EECPE. Following addition of 41.7 mg of unlabelled EECPE to the radioactive fraction isolated (41,000 d.p.m. ^{3}H; 4330 d.p.m. ^{14}C), 3 crystallizations were done from methanolwater. This resulted in the figures shown in Table 3. Insufficient material precluded further recrystallization.

Table 3.

Crystallization of radioactivity from fat with unlabelled EECPE.

Crystallization	Specific activity (d.p.m./mg) ^{3}H	^{14}C	$^{3}H/^{14}C$ Ratio
1	623	84	7.4
2	683	93	7.35
3	702	90	7.8

DISCUSSION

The very slow excretion of the administered radioactivity indicates that EECPE, or some derivative thereof, is stored in the body of human subjects for a long period of time. In the case of subject A.P. traces of radioactivity were still detectable in the urine 127 days after administration of the dose. The physiological potential of a single dose of EECPE is therefore of long duration.

Since EECPE was recovered from fat 2 and 3 days after ingestion, it is probable that it is stored unchanged. In any case, the storage form is one which permits the slow release of ethynylestradiol, since this compound was rigorously identified in all the urine samples examined. The slow release of this estrogen would explain the clinical observations of J.A. This subject received the single oral dose of EECPE on day 19 of her menstrual cycle. She did not menstruate until day 55. Her next period was 47 days long, followed by one of 33 days, after which she resumed a normal 28-day cycle. No clinical observations were made on A.P., who was anovulatory, or on the hysterectomized subjects.

The results indicate that all the excreted metabolites retain

the ethynyl side chain. Metabolite 1, (Fig. 4) is the only metabolite which retains the ^{14}C label. It is not known whether the cyclopentyl group on this compound has been altered or whether it remains intact.

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SECTION D

URINARY OESTROGEN CONJUGATE PATTERNS BASED MAINLY ON DIRECT HYDROLYSIS PROCEDURES

CONJUGATION OF URINARY PHENOLIC STEROIDS IN THE NONPREGNANT HUMAN FEMALE WITH PARTICULAR REFERENCE TO ESTRONE SULFATE

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Conjugation of Urinary Phenolic Steroids in the Nonpregnant Human Female with Particular Reference to Estrone Sulfate[1]

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ABSTRACT. The mode of conjugation of urinary metabolites of injected 17β-estradiol-6,7-^{3}H was studied in 10 nonpregnant human females. Estrone was the major identified constitutent of a fraction released from conjugation by solvolysis and this "estrone sulfate" accounted for 2–65% of total urinary estrone. In one instance, in an apparently normal young female, estrone-3-sulfate was directly identified as a quantitatively major metabolite, exceeding the glucosiduronates of both estrone and estriol. The considerable "sulfate conjugation" of estrone and of other estrogen metabolites in this subject was apparently a reproducible finding. Besides estrone, 2-methoxyestrone was regularly identified in the subjects studied, as a component of the fraction released by solvolysis and this conjugated form accounted for up to 63% of total urinary 2-methoxyestrone. Endogenous urinary "estrone sulfate" in the normal menstrual cycle varied considerably between subjects and accounted for 0–71% of total estrone. Other metabolites of labeled 17β-estradiol, including the latter itself, 16α-hydroxyestrone, 16-ketoestradiol-17β, 16-epiestriol and estriol, were less frequently identified following solvolysis of urine originally incubated with β-glucuronidase, *i.e.*, as "sulfates" and/or double conjugates such as "sulfoglucosiduronates." In 3 out of 11 studies 16α-hydroxyestrone and 16-ketoestradiol-17β were identified in this form (16–52% of total) but estriol conjugated in this way formed no more than 7% of total estriol. *J Clin Endocr* **29:** 328, 1969)

AN INCREASING interest in estrogen conjugates, including the sulfates, has been apparent over the years. In 1938 estrone sulfate[3] was identified as a constituent of pregnant mares' urine (1) and in the following year was shown to be present in human pregnancy urine (2). More recently, detailed information has become available on the mode of conjugation of certain urinary estrogen metabolites following injection of labeled precursors into the nonpregnant human female (3–5). However, there appears to be little knowledge as to the general pattern of individual phenolic steroid conjugates to be expected in the nonpregnant female. The present study was undertaken with a view to ascertaining the significance or otherwise of estrone sulfate in the urine of the nonpregnant female, both as an endogenous compound and as a metabolite of injected 17β-estradiol-6,7-^{3}H (^{3}H-E_2). During the course of the investigation some information was also obtained as to the conjugation of urinary 2-methoxyestrone (2 MeO-E_1), estradiol-17β (E_2), 16α-hydroxyestrone (16αOHE_1), 16-ketoestradiol-17β (16KE_2), 16-epiestriol (16epiE_3) and estriol (E_3).

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[3] The following abbreviations and trivial names have been used for steroids: 2-methoxyestrone (2MeO $-E_1$) = 2-methoxy-3-hydroxyestra-1,3,5(10)-trien-17-one; 16α-hydroxyestrone (16αOHE_1) = 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one; 16-ketoestradiol-17β (16KE_2) = 3,17β-dihydroxyestra-1,3,5(10)-trien-16-one; 16-epiestriol (16epiE_3) = estra-1,3,5(10)-triene-3,16β,17β-triol; E_1 = estrone; E_2 = 17β-estradiol; E_3 = estriol; estrone-3-sulfate = 3-yl-sulfate-estra-1,3,5(10)-trien-17-one. G = fraction extractable with diethyl ether following incubation with β-glucuronidase; S = fraction extractable with ether following solvolysis (after pre-extraction of G fraction) *or* fraction extractable with ether following incubation with phenolsulfatase.

Subjects

Subjects injected with 17β-estradiol-6,7-^{3}H. Ten females were studied. Six of these (MOL, 29 yr; VA, 40; TR, 25; MO, 42; SO, 23; GZ, 42) had normal cycles as judged by biphasic temperature curves, urinary pregnanediol levels and endometrial biopsies. MOL and VA were studied only at about the time of ovulation, ^{3}H-E_2 being injected at about day 12 of the cycle. MO, SO and GZ were injected in the

luteal phase (about day 19). TR was studied on 2 separate occasions, in each half of the cycle, at an interval of about 22 months. Two subjects (CO, 25 yr; PI, 23) were suffering from amenorrhea at the time of injection. Two others (OD, 57 yr; RO, 60) were post menopausal. None of these subjects was on any type of medication and no evidence was obtained for any endocrine, hepatic, renal or cardiac disorders.

Subjects in whom endogenous E_1S and E_1G were measured. This group consisted of 10 females (20–41 yr) exhibiting normal and regular cycles. One of these was studied on 2 occasions, some 3 months apart. No subject was on medication at the time of study and no clinical disorders were present. Measurements were made on 24 hr urines collected at about days 14 and 22 of the same cycle in each case.

Materials and Methods

Radioactive compounds. During the course of the study 2 batches of ^{3}H-E_2 were used. One, of specific activity (SA) 40 μCi/μg, was purchased from Merck, Sharp & Dohme Ltd., and was purified as described in an earlier publication (6). The second, of SA 20 μCi/μg, was obtained from New England Nuclear Corp., Boston, Mass., and was purified in the same manner. Estrone-6,7-^{3}H (^{3}H-E_1), of SA 10 μCi/μg, was purchased from New England Nuclear Corp. This was chromatographed on a Celite partition column (7) and the eluted material shown to be at least 99% pure by crystallization with the unlabeled form of the steroid. These labeled estrogens were stored in benzene: methanol (1:1) at −15 C and their purities checked at suitable intervals by chromatography and reverse isotope dilution.

Estrone-6,7-^{3}H-3-sulfate (SA 8 μCi/μg) in the form of its NH_4^+ salt was purchased from New England Nuclear Corp. This material was chromatographed on a 20 g column of aluminum oxide (Woelm, neutral, activity grade I) in methanol containing a small concentration of NH_4OH (2–3 drops conc. NH_4OH/100 ml). Radioactivity with the mobility of estrone sulfate (fractions 5–8; each 10 ml) was collected and the purity of the material checked by reverse isotope dilution with authentic unlabeled estrone-3-sulfate (see below). The results were confirmed by conversion to free estrone and then to estrone-3-acetate with crystallization at each stage. The data showed that the labeled estrone sulfate was about 99% pure. It was stored in methanol with the addition of 1–2 drops of conc. NH_4OH at −15 C and its purity was checked periodically.

n-Hexadecane-1,2-^{3}H (original SA 2.46 μCi/g) was purchased from The Radiochemical Centre, Amersham, Bucks., England. This was used for the preparation of absolute counting standards and was stored at 4 C. During the course of the study the half-life employed in calculating radioactive decay was 12.26 yr.

Nonradioactive steroids. All unlabeled steroids in their free forms were purchased from Mann Research Laboratories, Inc., New York. The purity of these was checked by thin-layer chromatography (8) and by melting point determination (microscope hot stage). Unlabeled estrone-3-sulfate (Na^+ salt) was synthesized in the laboratory by the method of Fieser (9). Extraction and purification was performed as described elsewhere (10). The NH_4^+ salt was prepared by solution in M NH_4OH and was reextracted with *n*-butanol. The crystalline material exhibited an infrared spectrum (KBr disc) identical with that of estrone-3-sulfate and on heating decomposed and finally sublimed at 230–233.5 C. On solvolysis (11) or hydrolysis with phenolsulfatase (12) it gave a quantitative yield of E_1. It was stored, in the dry state, for up to 3 weeks in an evacuated desiccator over anhydrous $CaSO_4$ at −15 C without apparent structural alteration.

Other chemicals and reagents. Bacterial β-glucuronidase powder (Type II) was obtained from Sigma Chem. Co., St. Louis, Mo., and Mylase P, containing sulfatase, was purchased from Mann Research Laboratories, Inc. Aluminum oxide, Woelm, activity grade I, was obtained from Alupharm Chemicals, New Orleans, and was stored in tightly stoppered bottles. Acetic anhydride was refluxed with fused sodium acetate and distilled twice, under anhydrous conditions, before use. Pyridine was similarly treated but with barium oxide. Tetrachloroethane and *p*-nitrophenol were treated as described elsewhere (13), as were Celite and all organic solvents used for extraction and chromatography (14). Sodium borohydride powder was stored in a desiccator over calcium chloride. Phosphors, for liquid scintillation spectrometry, are described in a later section of this paper.

Injection of subjects and urine collection. Purified ^{3}H-E_2 (about 15 μCi) was injected into an arm vein in 10 ml of 10% absolute ethanol in physiological saline. The dose given in each case was corrected for the radioactivity remaining in the syringe and vial following injection. Urine was collected in polyethylene bottles without preservative for 4×24 hr periods in each case. The

collections were either immediately processed or frozen at −15 C until required.

Where endogenous estrone was to be measured total 24 hr urines were collected and stored under the above conditions.

Hydrolysis, extraction and separation of radioactive metabolites. Suitable volumes of 4-day urine pools ($\frac{1}{2}$–$\frac{3}{4}$ of total volume depending on content of radioactivity, volume, etc.) were extracted with ether to remove unconjugated estrogens (<1% of the dose). The residual urine, from which excess ether had been removed, was adjusted to pH 7 with N NaOH or glacial acetic acid and buffered with 1/10 vol of 0.1M KH_2PO_4-NaOH buffer, pH 7. This was incubated for 24 hr at 38 C with 20 U/ml of bacterial β-glucuronidase (15). A further addition of the same amount of enzyme was followed by a second 24 hr incubation. Radioactivity released by this process was extracted with ether, the extract was washed with 1/10 vol of M $NaHCO_3$, then with 1/20 vol of H_2O and finally dried over anhydrous Na_2SO_4 prior to evaporation to yield a "glucosiduronate" (G) fraction. The residual urine was adjusted to pH 2 with 7N H_2SO_4, made 3M with NaCl and extracted with 3 vol of ethyl acetate. The steroid sulfates were solvolyzed at 38 C with H_2SO_4 as described by Segal *et al.* (11). The radioactive material so released was termed the "sulfate" (S) fraction.

The G and S fractions were separately but identically fractionated into their constituent labeled steroids by a procedure described earlier (6). This involved Girard separation, liquid/liquid partition and Celite partition column chromatography. Following the latter, 2MeO-E_1 and E_1 fractions were further purified by alkaline treatment and re-extraction (6). The sole deviation from the original procedure concerned the column ring D α-ketolic fraction (containing 16αOHE_1 and 16KE_2), which in this study was directly reduced with $NaBH_4$ (16). This yielded the triols, 16epiE_3 mainly from 16KE_2 (but also from 16β-hydroxyestrone) and E_3 mainly from 16αOHE_1, which were extracted from aqueous solution with ether, partitioned between toluene and N NaOH, re-extracted from alkali with ether following pH adjustment, and finally washed with M $NaHCO_3$ and H_2O prior to separation into 16epiE_3 and E_3 fractions on a Celite partition column (7).

Individual column eluates, or fractions thereof, of known radioactivity content were diluted with weighed amounts (10–30 mg depending on the radioactivity available) of the appropriate pure unlabeled steroids and crystallized from methanol until 2 successive SA values for the crystals agreed within the experimental error and matched the values for the corresponding mother liquors (ML). Radiochemical homogeneity was confirmed by crystallization in the form of the acetate derivative from methanol:H_2O. Acetylation was performed overnight at room temperature in a mixture of equal volumes of pyridine and acetic anhydride. On occasion, other derivatives were prepared *e.g.*, in the case of 2MeO-E_1 in which crystallization was first carried out in the free form then after formation of the 3-acetate, and finally, following preparation of 2MeO-E_2-3,17-diacetate by reduction with $NaBH_4$ and re-acetylation. Melting points of the final crystalline derivatives were checked on a microscope hot stage.

The mathematical product of the weight of carrier steroid added and the final SA of the pure crystals (in terms of free steroid) in each case yielded a value for the amount of radioactivity in the form of that particular steroid. This value was related as a percentage to the total radioactivity (G+S) extracted. These final values were corrected for the average losses (ranging from 60% recovery for ring D α-ketols to 85% for E_1) known to occur for the various pure steroids through the different extraction, Girard and column partition stages. This was not considered to be of much importance for the more chemically stable steroids since the main object of the study was to compare the relation between G and S forms of the various metabolites. However, particularly in the case of the more labile and poorly recovered ring D α-ketols (14), such a correction allowed better comparison between the different metabolites, stable and otherwise.

Stability of estrone-3-sulfate during β-glucuronidase incubation. Since the above study depended upon differentiation between "glucosiduronates" and "sulfates" by the hydrolytic sequence β-glucuronidase followed by sulfatase it was necessary to ascertain the stability of compound such as estrone-3-sulfate during incubation with the β-glucuronidase preparation. A number of urines from normal females not injected with radioactivity were each incubated with 2.29×10^5 dpm of pure ^{3}H-estrone-3-sulfate and β-glucuronidase under the conditions described above for urines containing radioactive metabolites. Any radioactivity released from conjugation was extracted with ether and assayed. In some cases the residual urine was submitted to solvolysis (11) and the free ^{3}H-E_1 recovered was estimated as a percentage of added ^{3}H-estrone-3-sulfate.

Extraction and purification of urinary ^{3}H-estrone sulfate in the intact form. Urine from a subject injected with ^{3}H-E_2 was extracted with ether at pH 6.5–7 and then with ethyl acetate at neutral pH following solution of 20 g NaCl/100 ml of urine. This latter "sulfate" fraction was evaporated in the presence of added NH_4OH and then submitted to a simple partition in a separatory funnel between *n*-butanol and 0.1N NaOH. The butanol fraction was washed with a small volume of saturated NaCl solution, evaporated, and chromatographed on aluminum oxide as described above. The "estrone sulfate" fraction eluted was then chromatographed on a 75 g Celite partition column in toluene:*n*-butanol:0.1N NH_4OH (6:4:5) in the general manner described by Siiteri (17). Radioactivity of mobility similar to that of estrone-3-sulfate was pooled, evaporated, and crystallized with pure unlabeled estrone-3-sulfate (NH_4^+ salt). Radiochemical purity was confirmed by further crystallization as free E_1 and then as E_1-3-acetate as described above for commercial ^{3}H-estrone-3-sulfate.

Measurement of endogenous E_1G and E_1S. One-third aliquots of 24 hr urines were pre-extracted (pH 6.5–7) with ether, and purified ^{3}H-estrone-3-sulfate (2.29×10^5 dpm; <.02 μg) was added as internal standard after 20 g NaCl/100 ml had been dissolved in each urine. The "sulfate" fraction was extracted with ethyl acetate and purified by partition between *n*-butanol and 0.1N NaOH then by chromatography on aluminum oxide as described above. The radioactive fraction so obtained was incubated at pH 6 in 0.1M acetate buffer for 18 hr with 2.5 mg/ml of sulfatase-containing Mylase P (4). The steroid fraction released from conjugation was extracted with ether and the extract was purified by shaking with $NaOH/NaHCO_3$, $NaHCO_3$ and H_2O (18) prior to drying over anhydrous Na_2SO_4. Estrone was then separated via a Girard reaction (7) and finally by a Celite partition column in the system benzene:0.8N NaOH (18). The estrone fraction was collected and analyzed as described below.

The residual urine, following ethyl acetate extraction, was adjusted to pH <2 and extracted with 3 vol of ether:ethanol (3:1). Although no labeled estrone-3-glucosiduronate was available to check the efficiency of extraction, it was ascertained that 17β-estradiol-6,7-^{3}H-17-glucosiduronate (New England Nuclear Corp.) was quantitatively extracted by this procedure. In the absence of labeled estrone glucosiduronate a known amount of pure ^{3}H-E_1 (1.5×10^5 dpm, <.01 μg) was added to the extract as a means of partially correcting for losses, although not including losses during subsequent hydrolysis with β-glucuronidase. The ether:ethanol was evaporated to dryness and incubated at pH 7 for 24 hr with bacterial β-glucuronidase as described above. Free estrogen was extracted with ether and E_1 was separated and purified as described for E_1 released from urinary E_1S.

Duplicate column eluates (usually 10–20% aliquots) containing E_1 from urinary E_1S and E_1G were measured spectrophotometrically by a micromodification of the Ittrich procedure (19). The chromogen formed by heating with 0.9 ml 65% (v/v) H_2SO_4 was diluted with 1.1 ml H_2O and extracted with 1.5 ml tetrachloroethane containing 2% (w/v) *p*-nitrophenol. The optical density (OD) of the latter extract was measured at 506, 538 and 570 mμ in a Unicam SP 600 visible light spectrophotometer fitted with a condensing lens, using micro cells of 0.8 ml capacity and 20 mm light path. The OD at peak wavelength (538 mμ) was corrected by the method of Allen (20) and related to suitable standards of known E_1 content. A minimum of *ca.* 0.05 μg of E_1 can be measured in this way and the relationship between corrected OD and weight is linear up to at least 1 μg. Aliquots of each column eluate were assayed for ^{3}H content to yield recoveries of added ^{3}H-estrone-3-sulfate and ^{3}H-E_1, and these values were used to correct for methodological losses. Recovery of ^{3}H-E_1 from added ^{3}H-estrone-3-sulfate was 70–80%. Recovery of ^{3}H-E_1 added to partially correct for losses during extraction and purification of E_1 from E_1G was 75–85%.

By this technique the lower limit of sensitivity for the measurement of E_1 originating as urinary E_1S was about 0.8 μg/24 hr, and for E_1 from urinary E_1G, about 1.5 μg/24 hr.

In order to obtain additional evidence for the identity of the endogenous material measured spectrophotometrically as E_1, suitable aliquots of column eluates (where E_1 was sufficiently high) were rechromatographed on 15 cm Celite columns in the solvent system *n*-hexane:benzene:70% methanol (1:1:2) (7), collecting 3 ml eluate fractions. ^{3}H content and E_1 weight were determined in each fraction and plotted against fraction number. A close correspondence of points was accepted as further proof of identity.

Measurement of radioactivity. In all cases this was performed by liquid scintillation spectrometry in 20 ml screw-cap glass vials (Wheaton Glass Co., Millville, N. J.).

TABLE 1. Purified urinary labeled metabolites (% of total G+S extract) following injection of ^{3}H-E_2

Subject	Steroid fraction													
	2MeO-E_1		E_1		E_2		16αOHE_1		16KE_2		16epiE_3		E_3	
	G	S	G	S	G	S	G	S	G	S	G	S	G	S
MOL	4.0	0.37	9.5	0.42	8.4	*	5.2	**	3.4	**	3.0	*	14.1	*
VA	3.6	<0.1	18.2	0.75	7.3	*	3.8	**	5.2	**	2.4	*	10.7	*
TRI (June '66)	0.83	0.64	9.9	7.1	7.3	*	4.3	1.2	3.5	2.5	4.0	*	26.2	*
TRII (April '68)	0.40	0.69	10.1	18.9	8.9	1.8	2.0	1.0	2.0	2.2	2.7	0.68	14.7	1.1
MO	2.3	1.3	14.5	2.7	7.5	*	4.0	**	3.5	**	2.6	*	11.7	*
SO	7.5	1.2	15.5	4.5	3.6	*	1.1	**	0.80	**	1.4	*	4.3	*
GZ	4.9	0.33	19.2	0.97	†	*	4.0	**	3.2	**	3.4	*	11.9	*
CO	1.6	0.25	13.4	0.90	10.2	0.5	2.5	0.48	1.5	0.86	4.2	0.66	25.0	1.0
PI	1.6	1.0	15.1	1.1	5.5	*	——lost——		——lost——		4.6	*	16.4	*
OD	3.2	<0.3	15.2	0.34	6.1	*	1.7	**	2.6	**	2.5	*	17.5	*
RO	1.7	<0.7	18.0	1.3	5.6	*	4.3	**	4.6	**	1.5	*	19.8	*
Average (G+S)	3.4		20.7		7.3		3.6		3.6		3.1		16.0	

* Radioactivity was so low as to exclude positive identification.

** Radioactivity was low, amounting, even before rigorous purification, to no more than 10% of the purified 16αOHE_1G and 16KE_2G fractions.

† No accurate value was obtained due to a very large decrease in SA on crystallization.

Total urinary radioactivity was counted in a dioxan scintillation medium [770 ml dioxan, 230 ml ethanol, 134 g naphthalene, 10 g 2,5-diphenyloxazole (PPO) and 250 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP)]. Urine extracts, column eluates, crystals, etc., were counted with or without the addition of methanol, as the occasion demanded, in toluene containing either 1 g/l of PPO and 0.3 g/l of POPOP or 7 g/l of butyl PBD [butyl derivative of 2-phenyl-5-(4-biphenylyl)-oxadiazole-1,3,4; Nuclear Chicago Corp.]. Quench correction was made by the internal standard procedure and all counts were converted to dpm. Counting was performed on a Nuclear-Chicago 6725 or 6850 spectrometer. Except in some cases for urine radioactivity sufficient counts were accumulated to ensure a counting error of no more than $\pm 3\%$.

Results

Following the injection of ^{3}H-E_2 the average excretion of radioactivity in the urine in four days was 63% of the dose. Of this an average of 50% of the urinary ^{3}H was extractable by the methods used. In every case the G fraction contained more ^{3}H than did the S fraction, but the G:S ratio with respect to radioactivity varied widely, from 1.3, in the case of subject TR on one occasion, to 10, for subjects MOL and GZ. The average G:S ratio was 6.5.

Table 1 contains data for individual metabolites in the G and S fractions as a percentage of the total extract (G+S) in each case. All of the values were based on rigorously purified fractions as outlined in the Materials and Methods section. E_1 was quantitatively the most important of the steroids identified in the S fraction. Also, although not a major metabolite, 2MeO-E_1S accounted for a very considerable percentage of total 2MeO-E_1. E_1 and to a lesser extent 2MeO-E_1 were the only steroids consistently identified as components of the S fraction. E_1S as a percentage of total E_1 ranged widely from about 2 to 65%. It is noteworthy that subject TR, on two separate occasions, excreted the S form of these steroids to a considerable extent. Thus, in the first study (TRI) 2MeO-E_1S and E_1S accounted for 44 and 42% of total 2MeO-E_1 and E_1, respectively. In the second study (TRII) the respective values were 63 and 65%. In this latter case E_1S was the major urinary metabolite identified, exceeding both E_1G and total E_3. Table 1 also shows the infrequency with which the nonketonic metabolites, E_2, 16epiE_3 and E_3, could be identified in the S fraction. Thus, E_2S was found to account for 17 and 5% of total E_2 in subjects TRII and CO, respectively; E_3S was 7% of total E_3 on two occasions in

TABLE 2. Stability of estrone-6,7-^{3}H-3-sulfate (2.29×10^5 dpm) during incubation with urine +β-glucuronidase for 48 hours at 38 C and pH 7

Urine no.	Ether-soluble E_1		E_1 recovery after solvolysis	
	dpm $\times 10^{-4}$	% original	dpm $\times 10^{-4}$	% original
1	0	0	—*	—
2	0.45	2.0	—	—
3	0.53	2.3	—	—
4	0.16	0.7	21.6	94
5	0.065	0.3	19.5	85
6	0.58	2.5	20.4	89
7	0.62	2.6	—	—
8	0.29	1.2	22.1	96
9	0.38	1.6	—	—
10	1.02	4.4	—	—
11	0.81	3.5	19.5	85
Mean	0.45	1.9	20.6	90

* Not analyzed.

TR and about 4% in CO; 16epiE_3S was about 20% of total 16epiE_3 in subject TRII and 14% in CO. The ring D α-ketols, in the form of their major chemically reduced products, were identified as components of the S fraction only in those same two subjects. 16αOHE_1S was 22 and 33% of total 16αOHE_1 in two studies on TR, and 16% in CO. The respective values for 16KE_2S were 42, 52 and 36%. All of this suggests the occurrence of individual conjugation patterns.

In view of the variability of E_1S between subjects, as shown in Table 1, it became necessary to enquire into the possibility that this might in part be due to E_1S hydrolysis during β-glucuronidase incubation. The results in Table 2 indicate that a definite, though small (0–4.4%), degree of hydrolysis does result during this process. These results render unlikely the possibility that E_1S could have contributed significantly to the E_1G fraction in our studies. Moreover, 85 to 96% of added ^{3}H-estrone-3-sulfate could be recovered as E_1 following solvolysis (Table 2).

Table 3 contains data regarding the extraction and purification of labeled ^{3}H-estrone-3-sulfate from the urine of subject TRII after injection of ^{3}H-E_2. In the experiment, 2.44×10^6 dpm, out of a total urine radioactivity of 19.07×10^6 dpm, were shown to be in the form of estrone-3-sulfate. This corresponded to 13% of the urinary radioactivity, uncorrected for experimental loss. Table 4 provides details for the crystallization of this labeled estrone sulfate with authentic unlabeled carrier. The results leave no doubt that estrone-3-sulfate was a major urinary metabolite in the case of subject TR.

Table 5 shows levels of endogenous E_1G and E_1S, in terms of free E_1, during the normal cycle. Subject TR, the individual

TABLE 3. Purification of urinary ^{3}H-estrone-3-sulfate (subject TRII) following injection of ^{3}H-E_2

Stage of purification*	dpm per 96 hr urine
Ethyl acetate extraction, neutral, 20% NaCl	4.4×10^6
n-Butanol/0.1N NaOH	4.0×10^6
Al_2O_3 column	3.7×10^6
Celite partition column	3.2×10^6
Crystallization with estrone-3-sulfate and purification via E_1 and E_1-3-acetate	2.44×10^6 (uncorrected)
Total urine radioactivity	19.07×10^6

* For details see Materials and Methods section of text.

TABLE 4. Purification by crystallization of urinary ^{3}H-estrone-3-sulfate (subject TRII) following injection of ^{3}H-E_2

Purification step	Calculated SA (dpm/mg)	Crystallization no.	SA crystals	SA ML
Estrone sulfate column fraction, 403,800 dpm ^{3}H +40.42 mg carrier estrone-3-sulfate (NH_4^+ salt); crystallized from methanol-ether	10,000*	1	7,240	**
		2	7,544	7,590
		3	7,520	7,620
Solvolysis of last crystals →E_1; crystallized from methanol	10,240†	1	10,320	10,240
		2	10,500	10,100
Acetylation of last crystals →E_1-3-acetate; crystallized from methanol-H_2O	9,080†	1	9,110	9,000
		2	9,090	9,140
Melting point of final derivative = 123.5–126.5 C				

* Assuming 100% ^{3}H-estrone-3-sulfate.
** Not measured due to weight of colored impurities.
† Based on SA of preceding crystals.

already shown to produce estrone-3-sulfate as a major metabolite from injected ^{3}H-E_2 (Tables 1, 3) was studied during two cycles, and on days 8, 14 and 22 of the cycle showed a relationship between endogenous E_1G and E_1S similar to that for the exogenous forms. E_1S accounted for up to 71% of total urinary E_1. Certain other subjects also excreted E_1S as an important fraction of total E_1; *e.g.*, in ZA (luteal phase) E_1S accounted for 51% and in MI for 62 and 57% of total E_1 in the proliferative and luteal phases, respectively. As was the case for exogenous metabolites (Table 1), endogenous E_1S varied considerably in its quantitative significance from one subject to another. In the admittedly small group of subjects studied, E_1S as a percentage of total E_1 averaged 32 and 37% at about day 14 and day 22, respectively, of the normal cycle.

Fig. 1 and 2 show superimposed radioactivity and weight of endogenous E_1 in two eluted fractions (E_1 from E_1G and E_1S, respectively, both from subject TR, luteal phase, April 1968) prepared from urines as described in the Materials and Methods section after pure ^{3}H-E_1 and ^{3}H-estrone-3-sulfate, respectively, had been added as internal standards. Despite the small num-

TABLE 5. Endogenous E_1G and E_1S (as free E_1 in μg/24 hr) in urine during the normal menstrual cycle

Subject	Age (yr)	Day 14 of cycle (approx)		Day 22 of cycle (approx)	
		E_1G	E_1S	E_1G	E_1S
TR (Jan. 1968)	27	16.3	20.8	9.9	21.5
TR (April 1968)	27	5.1*	12.7*	12.1	29.1
SO	24	4.9	1.5	4.9	<0.2**
GA	24	20.8	2.2	11.4	3.7
CH	22	3.4	1.7	18.2	7.4
TY	20	6.6	0.9	3.7	0
GR	32	18.1	3.6	16.0	3.2
ME	25	12.0	4.7	8.6	1.0
ZA	29	8.4	3.8	4.6	4.8
MA	29	21.4	5.6	10.2	5.2
MI	41	5.5	9.0	6.1	8.2
Mean†		11.7	5.4	9.4	5.5

* Day 8 of cycle.
** Not significantly different from zero.
† Does not include TR (April 1968).

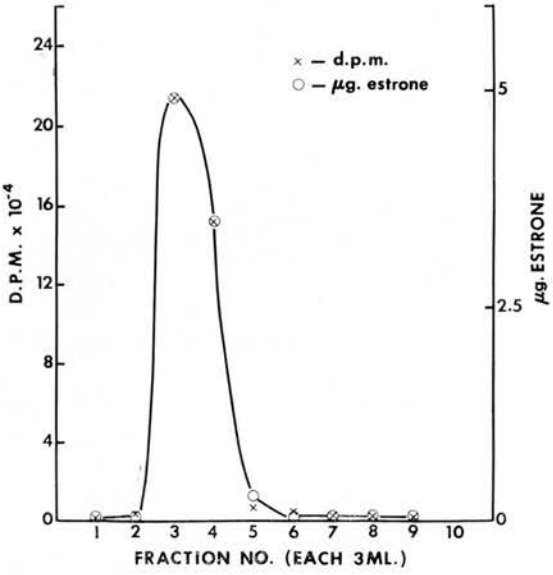

FIG. 1. Celite partition column chromatography of an E_1 fraction obtained from urinary E_1G, subject TR (April 1968), after the addition of pure ^3H-E_1 to the urine extract.

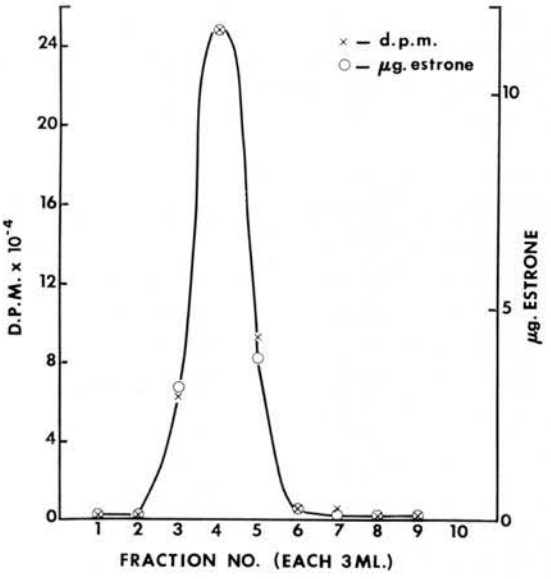

FIG. 2. Celite partition column chromatography of an E_1 fraction obtained from urinary E_1S, subject TR (April 1968), after the addition of pure ^3H-estrone-3-sulfate to the urine.

ber of points on each curve there is good agreement between weight and radioactivity with no extraneous Kober-positive material in the elution pattern. Also, recoveries of radioactivity and weight were similar in each case. It should be noted that a slight difference between the elution curves of Fig. 1 and 2 could be attributed to a temperature difference during chromatography. These data further indicate the identity of the ketonic, Kober-positive compound, purified and measured in this study, to be E_1.

Discussion

Several technical points in the study should be considered in relation to this discussion since, in view of the hydrolytic procedures adopted, absolute certainty regarding the identity of the individual conjugates is lacking, except where urinary ^3H-estrone-3-sulfate was directly crystallized with carrier. In the case of labeled 2MeO-E_1G and E_1G fractions it seems more than likely that these would indeed represent the 3-glucosiduronates of the respective steroids and would not be contributed to, to any great extent, by the sulfate forms. Nevertheless, it should not be overlooked that some β-glucuronidase preparations will hydrolyze the linkage between steroids and N-acetylglucosamine (21). However, to date there is no reason to suspect the presence of important amounts of urinary E_1 or 2MeO-E_1 in such a conjugated form. Available evidence is likewise in favor of the radioactive fractions termed 2MeO-E_1S and E_1S in this study being the 3-sulfates of these steroids. Steroids bearing more than one hydroxyl group, however, pose an uncertainty, particularly where the S fraction is concerned. Thus, solvolysis following β-glucuronidase action may release steroids originally conjugated as sulfate (22), sulfoglucosiduronate (4) or even perhaps doubly conjugated with sulfuric acid and N-acetylglucosamine (21), although the available evidence suggests that little of the latter may appear in the urine (23). It is not possible in the present study to differentiate between these possibilities where E_2S, 16epiE_3S, E_3S and ring D α-

ketols S are concerned. Also, it may be that minimal detection has been made of doubly conjugated forms because of the hydrolytic sequence used above since the reverse (*i.e.*, sulfatase followed by β-glucuronidase) procedure is claimed to be more effective, at least for the hydrolysis of E_3-3 sulfate-16-glucosiduronate (24).

It must also be remembered that, whereas the values reported for endogenous E_1S have been corrected for all methodological losses, the E_1G fraction was only corrected for such losses occurring following conjugate extraction and hydrolysis. Thus, E_1S related to "total E_1" would yield a maximal figure. However, it is unlikely that this would result in a major error since the degree of extraction, as judged using 17β-estradiol-17-glucosiduronate, was complete, and furthermore, available evidence suggests efficient hydrolysis of phenolic steroid 3-glucosiduronates by β-glucuronidase preparations (15, 25).

Bearing in mind the above limitations of the methodology employed, there can be little doubt that estrone-3-sulfate may be on occasion a quantitatively significant urinary metabolite, exogenous or endogenous, in the nonpregnant human female. Thus, in one apparently normal subject (TR) this metabolite, from injected ^3H-E_2, exceeded even E_1G and E_3G. Moreover, the quantitative significance of S conjugation in this subject appeared to be a reproducible finding at intervals over a period of almost two years. This finding related to the conjugation of all the phenolic steroids studied and suggests the necessity for further investigation into the reasons for such an individual pattern; *e.g.*, the part played by the kidney.

Although E_1 was the main identified steroid component of the urinary S fraction following ^3H-E_2 injection, it is clear that E_1S in relation to E_1G, whether endogenous or exogenous, may show a considerable variation and, on the average, E_1G is greater. McKenna *et al.* (26) have shown that some 8% of the E_1 in pooled late pregnancy urine may be conjugated with sulfuric acid. Unpublished work from our laboratory suggests a higher figure although variation between subjects is as wide as has been found for nonpregnant females in the present study. It should also be pointed out that sulfate conjugation of urinary E_1 may be quantitatively important in the human male (27, 28). The only steroid besides E_1 more or less regularly identified in the S fraction during the present investigation was 2MeO-E_1. Here again the individual variation was very wide but it is interesting to note that the two steroids most consistently S conjugated (almost certainly sulfate) are both ketonic in nature.

When compared with the case of 2MeO-E_1 and E_1 the identification of E_2S, 16epi-E_3S, E_3S, 16αOHE_1S and 16KE_2S was seldom achieved in this study. It could be argued that injection of a larger dose of ^3H-E_2 might have allowed more frequent detection of these forms. This could well be so but it would not alter the relative differences in conjugation noted above. Also, it is possible that the S forms of the ring D α-ketols are less stable during solvolysis than is E_1S or 2MeO-E_1S. However, this would not be likely to account for the lack of stable steroids such as E_3 and E_2 in the S fraction. Although the ring D α-ketols, particularly 16KE_2 (in its reduced form and therefore possibly partly due to 16β-hydroxyestrone), were identified to a significant extent in the S fraction, even if only in three subjects, E_3S accounted for no more than 7% of total E_3 in any case. Whether these conjugation differences can be attributed to such factors as specific tissue enzyme activities, renal handling, plasma or tissue protein binding, is not clear, and the answers must await further work.

The pattern of urinary metabolites (aside from the question of conjugation) of injected ^3H-E_2 appears generally similar to that found by other investigators (29, 30). In the present study E_1 was on the average the main steroid identified, with E_3 a close second. An average of 58% of the G+S

urinary extract has been accounted for in the form of identified steroids, which seems reasonable since no attempt was made to investigate the quantitatively important 2-hydroxyestrone [2,3-dihydroxyestra-1, 3,5(10)-trien-17-one] (30). The urinary ring D α-ketols ranged from 1.9 to 11.5% of the total extract and thus appear to be less quantitatively significant than originally thought on the basis of studies in our laboratory (16). This finding is presumably due to the more rigorous purification procedures employed in the present study than in earlier work.

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CONJUGATION OF PHENOLIC STEROID RING Dα-KETOLS IN HUMAN PREGNANCY URINE

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SUMMARY

Sequential solvolysis, and hydrolysis with β-glucuronidase followed by suitable separation and purification steps has provided some data on the conjugation of phenolic steroid ring Dα-ketols in late pregnancy urine as compared with the pattern for estriol. In 6 experiments 16α-hydroxyestrone was found to be, on the average, 80% in a form hydrolyzable by β-glucuronidase, 2% in a form split by solvolysis (presumably sulfate) and 18% in a form behaving on hydrolysis like a sulfoglucosiduronate. The corresponding values for 16-ketoestradiol-17β (including perhaps some 16β-hydroxyestrone) were 67%, 18% and 15% and for estriol, 94%, 2% and 5%. In 12 experiments the average "non-glucosiduronate" forms of 16α-hydroxyestrone, 16-ketoestradiol-17β and estriol amounted to 21%, 38% and 5% of each, respectively. The "non-glucosiduronate" conjugates of estriol were, by weight, generally greater in amount than those of the ring Dα-ketols. Qualitative supporting evidence for the presence of these ring Dα-ketol conjugates was obtained by direct DEAE-Sephadex and Celite partition chromatography followed by suitable enzyme hydrolysis.

INTRODUCTION

Isolation from pregnancy urine of 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one (16α-hydroxyestrone; $16\alpha OHE_1$), 3,17β-dihydroxyestra-1,3,5(10)-trien-16-one (16-ketoestradiol-17β; $16KE_2$) and 3,16β-dihydroxyestra-1,3,5(10)-trien-17-one (16β-hydroxyestrone; $16\beta OHE_1$) has been reported[1,2]. Little information has appeared regarding the conjugation pattern of these ring Dα-ketols although this group of steroids is quantitatively important in pregnancy[2-4]. Isolation experiments have shown that as much as 50% of urinary $16\alpha OHE_1$ may be doubly conjugated, with

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sulfate at C-3 and glucosiduronate at C-16 of the steroid molecule (*i.e.* 16αOH$E_1$3S16G) (ref. 5). Also, the presence of a sulfate conjugate of 16KE_2 has been suggested[6]. The present report presents data on the conjugation of these steroids in comparison with that of estriol (E_3).

MATERIALS

Complete 24-h urines were collected during the third trimester of pregnancy and these were frozen immediately upon receipt in the laboratory until analyses could be performed.

All chemical reagents, including organic solvents and supporting materials for chromatographic columns and thin-layer plates, were obtained and purified where necessary by published methods[7,8]. Bacterial β-glucuronidase powder, type II, was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.,and Mylase P (containing phenosulfatase) from Mann Research Laboratories, Inc., New York, N.Y., U.S.A. These were used for hydrolysis of conjugates as described elsewhere[7].

Unlabelled steroids were purchased from Mann Research Laboratories, Inc., except for 16αOHE_1, which was a gift from Dr. A. E. Kellie, Courtauld Institute of Biochemistry, Middlesex Hospital, London, England.

[6,7-^{3}H]17β-Estradiol (^{3}H-E_2) of specific activity 40 C/mmole was purchased from New England Nuclear Corp., Boston, Mass., U.S.A., and was suitably purified prior to use[7]. [6,7-^{3}H]Estrone-3-sulfate (^{3}H-$E_1$3S), of specific activity 2.8 C/mmole, was purchased as the NH_4^+ salt from New England Nuclear Corp. and [4-^{14}C]estrone-3-glucosiduronate (^{14}C-$E_1$3G), of specific activity 27 C/mole, was biosynthesized in our laboratory[9]. These conjugates were purified by DEAE-Sephadex and Celite chromatography[9].

METHODS

Differential hydrolysis of urinary conjugates

Unconjugated estrogens (negligible amounts) were extracted from the urine with ether prior to conjugate hydrolysis in all experiments.

In 6 experiments utilizing urines from different subjects, separate urine aliquots (usually one-sixth of 24 h) were subjected to solvolysis to split steroid sulfates (S)[10] and to β-glucuronidase treatment to hydrolyze steroid glucosiduronates (G)[7]. The aliquots hydrolyzed with β-glucuronidase were extracted with ether to remove free steroids and the aqueous residues were subjected to solvolysis to yield a S+SG fraction, *i.e.* steroids arising from original urinary steroid sulfates together with those which were originally "doubly conjugated" and which had been partially split by β-glucuronidase to yield sulfates. Thus the value (S+SG)−S yielded a measure of "sulfoglucosiduronates" (SG).

In 6 further experiments, using urine from different subjects than those above, G and "non-G" (*i.e.* S+SG) fractions were obtained with no attempt to differentiate between S and SG.

Separation of hydrolyzed steroids

Following extraction the steroids were separated by a combination of the

Girard reaction, partition column chromatography, $NaBH_4$ reduction of the ring Dα-ketols and further chromatography of the steroidal triols so formed[3,7]. The separated steroid fractions were measured by an Ittrich–Kober spectrophotometric reaction[7,8] and further identification was obtained by thin-layer chromatography in comparison with standard steroids[8].

Recovery experiments

Known weights of the various pure steroids were added to human non-pregnancy urine, or to extracts at various points of the experimental procedure, and processed exactly as described for the urines above. This provided data on experimental losses during the separation and purification techniques.

Further identification of the ring Dα-ketol conjugates

In one experiment [^{14}C]$E_1$3G and [^{3}H]$E_1$3S were added to an aliquot (one-fifth of 24 h) of late pregnancy urine to act as internal standards. The urine was passed through Amberlite XAD-2 resin[11] and the conjugate fraction obtained was chromatographed in a concentration gradient of NaCl (0–0.8 M) on DEAE-Sephadex[9]. Fractions containing ^{3}H and others adjacent to these (*i.e.* fractions behaving like estrogen sulfates) were analyzed for steroid content following enzyme hydrolysis.

In 2 further experiments [^{3}H]E_2 was injected intravenously (arm vein) into each of 2 subjects during the last month of pregnancy. Urine was pooled in each case over the succeeding 48 h and an aliquot (one-sixth) of each pool was processed by XAD-2 resin and DEAE-Sephadex chromatography as above. ^{3}H-containing fractions with the mobility of sulfates and/or sulfoglucosiduronates were subjected to analysis on Celite columns in the solvent systems 1-butanol–ethyl acetate–0.2% NH_4OH (ref. 12) (system A) and iso-octane–*t*-butanol–MNH_4OH (ref. 13) (system B) before and after partial enzyme hydrolysis[9,14]. Following complete enzyme hydrolysis the fractions were analyzed for steroid content as described above.

RESULTS

Conjugation of steroids as revealed by direct differential hydrolysis

Table I contains data on the levels of three conjugated forms of E_3, 16αOHE_1 and 16KE_2. These values have been corrected for average losses occurring in the experimental procedure. This gives a more realistic picture since the overall recovery of ring Dα-ketols (including reduction to, and separation of, the triols) is only 44–56% while that of E_3 is 74–83%. It was found that 94% of E_3 was in the G form while only some 2% was conjugated as S. Also, 16αOHE_1 S was extremely low. However, 16KE_2 (probably 16KE_2+16βOHE_1 due to the methods used, although the latter is much smaller than the former[3]) appeared to be conjugated 18% on the average as S, while both 16αOHE_1 and 16KE_2 were found in the SG form (16αOHE_1 = 18%, 16KE_2 = 15%). E_3SG, although representing only 4% of total E_3, was by weight the main "non-G" form detected for any of the steroids measured.

Some support for the validity of the expression (S+SG)—S was obtained by measuring E_1 in these urines following solvolysis alone and after β-glucuronidase treatment with subsequent solvolysis. In each case application of the above expression

TABLE I

CONJUGATION OF RING Dα-KETOLS AND ESTRIOL AS G, S AND SG IN NORMAL PREGNANCY URINE (mg/24 h)

Expt.	E_3			$16\alpha OHE_1$			$16KE_2$		
No.	*G*	*S*	*SG*	*G*	*S*	*SG*	*G*	*S*	*SG*
1	10.5	0.18	0.30	0.89	0.04	0.48	0.56	0.23	0.32
2	14.3	0.22	0.93	1.4	0.02	0.30	0.93	0.14	0.27
3	14.3	0.22	2.0	6.7	0.04	1.8	3.4	0.45	1.3
4	37.1	0.46	1.2	2.4	0.18	0.59	2.3	0.82	*
5	36.2	0.63	1.4	4.7	0.18	0.46	2.9	1.1	0.27
6	38.8	0.97	0.83	2.0	*	0.48	1.5	0.34	0.32
Mean	25.2	0.45	1.1	3.0	0.08	0.70	1.9	0.51	0.41
Mean as % total for each steroid	94	2	4	80	2	18	67	18	15

* Steroid not detected in measurable amount.

gave a result differing insignificantly from zero, thus showing that E_1 (with only 1 OH group) was not being artifactually measured in the SG form.

When $16\alpha OHE_1$ was added to non-pregnancy urine in 4 experiments, subjected to solvolysis, and then to separation and purification including $NaBH_4$ reduction to the triols, 58% (52–63) was recovered in the E_3 fraction and 6% (5–8) in the estra-1,3,5(10)-triene-3,16β,17β-triol (16-epiestriol; 16epiE_3) fraction, *i.e.* typical of direct reduction of pure $16\alpha OHE_1$ (ref. 15). These recovery values were unchanged when solvolysis was omitted and the remainder of the procedure carried out. This showed the lack of a destructive effect of solvolysis itself on $16\alpha OHE_1$ and suggests that the low values for $16\alpha OHE_1S$ in the pregnancy urines studied (Table I) are meaningful.

Table II contains data for G and "non-G" conjugates in a further 6 pregnancy urines. E_3, $16\alpha OHE_1$ and KE_2 were conjugated on the average as "non-G" to the extent of 4, 22 and 42%, respectively. This compared with 6, 20 and 33% for the "non-G" conjugates of Table I. Thus for the 12 urines analyzed the average "non-G" conjugation of E_3, $16\alpha OHE_1$ and $16KE_2$ was 5, 21 and 38% respectively.

Additional identification of ring Dα-ketol conjugates

Following addition of [^{14}C]E_13G and [^{3}H]E_13S to an aliquot of pregnancy

TABLE II

CONJUGATION OF RING Dα-KETOLS AND ESTRIOL AS G AND S+SG IN NORMAL PREGNANCY URINE (mg/24 h)

Expt.	E_3		$16\alpha OHE_1$		$16KE_2$	
No.	*G*	*S+SG*	*G*	*S+SG*	*G*	*S+SG*
1	8.4	0.26	0.73	0.44	2.2	2.9
2	11.0	0.06	0.55	0.14	0.57	0.09
3	31.4	1.6	2.9	0.56	1.8	0.74
4	26.5	1.1	1.3	0.28	0.78	0.24
5	26.7	1.1	1.8	0.30	1.3	0.74
6	32.5	1.9	2.7	1.1	1.8	1.4
Mean	22.8	1.0	1.7	0.47	1.4	1.0
Mean as % total for each steroid	96	4	78	22	58	42

urine, well separated ^{14}C and ^{3}H peaks were obtained on DEAE-Sephadex chromatography. Fractions in the region of the ^{3}H eluted, *i.e.* associated with steroid sulfates (tubes 47–70, 10 ml fractions), were pooled and subjected to Mylase P hydrolysis yielding 166 μg of Kober-positive steroid, together with the ^{3}H label, in ether-soluble form (fraction I). Further hydrolysis with β-glucuronidase released 195 μg of steroid accompanied by no ^{3}H (fraction II). Fraction I consisted of E_3 (112 μg) together with a very small amount (7.5 μg) of ring Dα-ketols ($16KE_2$:$16\alpha OHE_1$ ratio = at least 4). Fraction II, probably sulfoglucosiduronate, gave on analysis 126 μg E_3 and 33 μg ring Dα-ketols (78% corresponded to $16\alpha OHE_1$ and the remainder to $16KE_2$). The close association of doubly conjugated ring Dα-ketols with the monosulfate fractions differed markedly from the clear separation found between 17β-estradiol-3-sulfate and 17β-estradiol-3-sulfate-17-glucosiduronate[9].

Following injection of [^{3}H]E_2 into one subject (C), DEAE-Sephadex chromatography yielded 2 broad peaks of radioactivity. The second of these (tubes 45–70) was analyzed as shown in Table III. A portion of this unhydrolyzed peak yielded one main peak (79% of applied ^{3}H) at holdback volume (HBV) 1–2 (typical of monosulfate) on Celite in system A. Rechromatography in system B resulted in a peak, containing 67% of applied ^{3}H, eluted over tubes 11–18 (E_13S eluted over 11–16).

TABLE III

ANALYSIS OF URINARY SULFATES AND SULFOGLUCOSIDURONATES AFTER INTRAVENOUS INJECTION OF [^{3}H]E_2
(Subject C)

Steroid fraction	*Hydrolysis by Mylase P*		*Subsequent hydrolysis by β-glucuronidase*	
	decomp./min ^{3}H	*μg*	*decomp./min ^{3}H*	*μg*
E_1	128000	192	*	*
Ring Dα-ketols	30000	130	22200	84
E_3	16000	102	8500	152

* Negligible radioactivity or weight.

In a second injection experiment (subject CV) 2 broad peaks of ^{3}H were again obtained from DEAE-Sephadex. The first of these corresponded to glucosiduronate while the second, partially separated into two (peak IIA, tubes 37–44, 16% of recovered ^{3}H; peak IIB, tubes 47–54, 12% of recovered ^{3}H) was in the region of monosulfate and possibly sulfoglucosiduronate. An additional small peak, peak III, containing 2% of recovered ^{3}H was present at tubes 55–80. Peaks IIB and III were each chromatographed on Celite in system A and in each case some ^{3}H was eluted in the region of monoconjugates. More polar material was eluted with methanol in each case and these fractions were combined for analysis as shown in Table IV. The data indicate that a considerable proportion of the material behaved as the sulfoglucosiduronates of $16\alpha OHE_1$ and $16KE_2$ with the former predominating.

DISCUSSION

Other investigators have applied the technique of differential hydrolysis to indicate the presence of mixed conjugates in urine[16] and in plasma[17]. Such a technique can scarcely be described as ideal, particularly when applied to the problem of the

TABLE IV

STEPWISE CELITE CHROMATOGRAPHY AND ENZYME HYDROLYSIS OF URINARY RING Dα-KETOL SULFOGLUCOSIDURONATES AFTER INJECTION OF [^{3}H]E_2
(Subject CV)

Fraction	*Purification step*
Polar fraction from Celite 75000 decomp./min ^{3}H, 86 μg steroid	System B eluted 26800 decomp./min ^{3}H, 55 μg steroid at 8 HBV*
½ fraction from system B	β-Glucuronidase → < 6% ether-soluble ^{3}H; aqueous residue in system A gave peak in fractions 14–22 (E_13S elutes in 11–16); subsequent Mylase P gave complete hydrolysis
½ fraction from system B	Mylase P → 6% ether-soluble ^{3}H; aqueous residue in system A gave peak in fractions 31–38 (E_23G elutes in 25–35); subsequent β-glucuronidase gave complete hydrolysis
Pooled free fractions after enzyme hydrolysis	Girard separation → 83% ^{3}H ketonic, 18% non-ketonic; ketonic fraction on Celite → 65% ^{3}H and 80% weight corresponding to ring Dα-ketols; $NaBH_4$ reduction then Celite chromatography → 64% recovered ^{3}H and 70% recovered weight in E_3 fraction (*i.e.* from 16αOHE_1), 36% ^{3}H and 30% weight in 16 epiE_3 fraction (*i.e.* from 16KE_2 ± 16βOHE_1).

* Typical of sulfoglucosiduronate. This fraction gave immediate pink colour in Ittrich–Kober reaction, typical of 16αOHE_1.

conjugates of the rather unstable ring Dα-ketols. However, supporting evidence for the results of the present study resides in the absence of E_1 from the SG fraction as described above, and in the conjugation pattern found for E_3 which is strikingly similar to that reported by others[18,19].

Although the "non-G" conjugated forms of E_3 appear to be present in greater amounts than those of the ring Dα-ketols, the percentage of the latter steroids in the form of "non-G" is much greater than for E_3. This is particularly striking with respect to 16αOHE_1SG, 16KE_2S and 16KE_2SG. It is noteworthy, however, that the S form of 16αOHE_1 is present in very small amounts indeed (average 0.08 mg/24 h) when compared with E_3S (0.45 mg/24 h) and 16KE_2S (0.51 mg/24 h). The reason for this finding is not clear at present. In any case it is evident that any procedure for the total hydrolysis of ring Dα-ketols conjugates in pregnancy urine must include a means of splitting sulfate conjugates.

The data obtained in this study by chromatographing and differentially hydrolyzing the urinary conjugates, while qualitative at best, suggests that where sulfate groups are found these are at the C-3 position, in view of the hydrolysis achieved by phenol-sulfatase. In the doubly conjugated forms, therefore, it is likely that the glucuronic acid moiety is attached to ring D. The position of the glucuronic acid in the ring Dα-ketol glucosiduronates is at present unknown.

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SECTION E

FORMATION, METABOLISM, INTERCONVERSION OF OESTROGEN SULPHATES IN SPECIES OTHER THAN THE HUMAN IN VITRO AND IN VIVO

BIOSYNTHESIS OF STEROID SULFATES BY A RAT LIVER PREPARATION[1]

H. R. RAUD[2] AND R. HOBKIRK

Within recent years it has been indicated that biosynthesis of steroid sulfates may be important in phenomena other than detoxification. Thus, attention has been drawn to the interconversion of certain steroids in the form of sulfates (1, 2), the secretion of dehydroisoandrosterone (DHA)[3] sulfate by the adrenal cortex (3), the importance of estrone sulfate in blood (4), and the quantitative significance of estrogen sulfate formation by the human fetus (5). The present report contains information on the activity of a soluble fraction from rat liver with respect to sulfurylation of certain phenolic steroids and the manner in which the age of an animal affects this conjugation.

Materials and Methods

16-KE_2-16-^{14}C (44 μcuries/mg) and estriol-16-^{14}C (22 μcuries/mg) were purified by column, paper, or thin-layer chromatography to yield material of at

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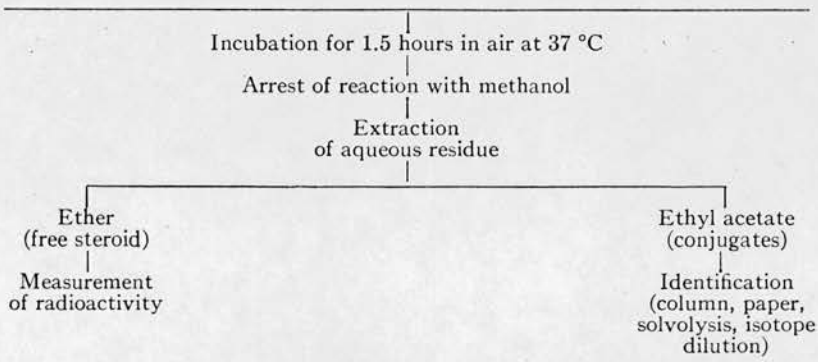
[3]The following trivial names and abbreviations are used in the text: DHA = dehydroisoandrosterone (3β-hydroxy-Δ^5-androsten-17-one); 16-KE_2 = 16-ketoestradiol-17β (3,17β-dihydroxy-estra-1,3,5(10)-trien-16-one); 16-epiestriol = 3,16β,17β-trihydroxy-estra-1,3,5(10)-triene; estriol = 3,16α,17β-trihydroxy-estra-1,3,5(10)-triene; estrone = 3-hydroxy-estra-1,3,5(10)-trien-17-one; estradiol-17β = 3,17β-dihydroxy-estra-1,3,5(10)-triene; ATP = adenosine triphosphate.

least 98% purity. 16-Epiestriol-16-^{14}C was prepared by chemical reduction of 16-KE_2-16-^{14}C as described elsewhere (6). Estradiol-17β-6,7-^{3}H (New England Nuclear Corp.) of specific activity 150 μcuries/μg was purified by column partition chromatography and shown to be at least 98% pure by reverse isotope dilution. Estrone-16-^{14}C (Radiochemical Centre, Amersham, England) (45 μcuries/mg) and DHA-4-^{14}C (Merck Sharp & Dohme Ltd.) (140 μcuries/mg) were judged to be at least 99% pure after thin-layer chromatography. ^{35}S-labeled inorganic sulfate (New England Nuclear Corp.) (1.3 mcuries/mg) was used as obtained. ATP was purchased as the disodium salt from Sigma Chemical Company, St. Louis, Mo. All other chemical reagents were purified, when necessary, by standard procedures. Estrone and estradiol-17β sulfates were synthesized as described by Fieser (7). The 3-methyl ethers of estrone and estradiol-17β were prepared by the method of Brown (8).

A supernatant fraction (20,000 $\times$ *g*) was obtained from the pooled livers of white Wistar rats as described by Bernstein and McGilvery (9). This was fractionated with $(NH_4)_2SO_4$, and the material precipitating between 1.7 *M* and 2.3 *M* salt concentration was used as the source of enzyme in all experiments. The procedure of incubation, extraction, and product identification is outlined in the following scheme.



Incubation mixture: 0.1 ml (0.04 μmole) labeled substrate (ca. 20,000 c.p.m.) in propylene glycol; 0.5 ml buffered ATP, pH 6.8 (1 volume 0.3 *M* KH_2PO_4, 1 volume 0.03 *M* K_2SO_4, 1 volume 0.005 *M* $MgCl_2$, 0.33 volume 0.08 *M* ATP disodium salt); 0.4 ml enzyme preparation (equivalent to ca. 100 mg wet liver tissue).

Aliquots of all extracts were assayed for radioactivity by liquid-scintillation spectrometry. An average of 81 $\pm$ 3.5% of the incubated radioactivity was recovered in ether and ethyl acetate extracts. Because of a lack of pure steroid sulfates of certain types, rigorous identification studies (i.e. crystallization) were made only upon the incubation products of estrone, estradiol-17β, and DHA. In the case of the product of estradiol-17β incubation in particular, celite partition column chromatography was carried out in the solvent system isooctane – *t*-butanol – 1 *M* NH_4OH (200:500:500) (10). The values for percentage conjugation of estriol, 16-epiestriol, 16-ketoestradiol-17β, and estradiol-

17β-3-methyl ether were obtained from the amount of radioactivity behaving as sulfate conjugate during paper chromatography in toluene – *n*-butanol – 2.8% (v/v) NH_4OH (3:1:2) (11), and subsequently associated with each of the free compounds after solvolytic cleavage (12).

Results and Discussion

Table I contains data on the extent of estrogen sulfate formation by the enzyme preparation from livers of adult female rats compared with the

TABLE I

Steroid sulfate synthesis by the enzyme preparation from adult female rat liver*

Steroid substrate	% conjugation of substrate
16-^{14}C-estrone	6.4±0.7
6,7-^{3}H-estradiol-17β	12.7±0.4
16-^{14}C-estriol	3.5±0.2
16-^{14}C-16-epiestriol	9.8±0.7
16-^{14}C-16-ketoestradiol-17β	6.0±0.3
6,7-^{3}H-estradiol-17β-3-methyl ether	14.8±0.7
4-^{14}C-dehydroisoandrosterone	68.8±1.4

*Figures are averages from at least three experiments.

synthesis of DHA sulfate. At the substrate concentrations studied, conjugation of DHA was considerably greater than that of any of the phenolic steroids incubated. However, detectable conjugate formation was seen in all cases, particularly of those estrogens possessing β-orientated hydroxyl groups in the D ring, namely, estradiol-17β and 16-epiestriol. As yet insufficient information has been obtained to show the significance of this as a purely structural effect.

Solvolytic cleavage of the incubation products resulted in the release, in ether-soluble form, of about 90% of the radioactivity present in the conjugated fractions. Paper chromatography also showed these products to have mobility similar to that of the standard steroid sulfates available. Dilution of the labeled product of estrone incubation with unlabeled estrone sulfate to give a calculated specific activity of 547 c.p.m./mg was followed by crystallization from methanol–ether mixture. On successive crystallizations the crystals showed a specific activity of 536, 526, and 511 c.p.m./mg; the respective mother liquors had values of 525, 538, and 527 c.p.m./mg. This indicated that most of this material was estrone sulfate. The similar degree of conjugation of estradiol-17β and its 3-methyl ether (Table I) suggested that the former might be conjugated only at C-17 by the enzyme preparation. However, incubations in which ^{35}S-labeled inorganic sulfate was used resulted in the formation of conjugated estradiol-17β with a ^{35}S/^{3}H molar ratio of 2.05, compared with a value of 1.03 for the 3-methyl ether of this compound. Moreover, partition column chromatography of the estradiol product showed it to have the mobility of estradiol-3,17β disulfate; and dilution of this same material with the 3-monosulfate and the 17-monosulfate of estradiol-17β, separately, then crystallization, yielded virtually no radioactivity in the crystals. Thus, under the

experimental conditions employed, it would appear that the product was the 3,17-disulfate of estradiol-17β. Wengle and Boström (13) reported the formation of both the disulfate and monosulfate of estradiol-17β when unfractionated cell-free rat liver systems were employed.

Table II contains data on the effect of the age of the animal on the sulfurylating activity of the same enzyme system. It is obvious that such activity is

TABLE II

Effect of age of animal on steroid sulfate synthesis by the liver enzyme preparation*

	% conjugation of substrate		
Age of animal	Estrone	Estradiol-17β	Dehydroisoandrosterone
Fetus (term)†	0	0	2.8±0.9
10 days†	0	1.6±0.4	13.1±0.7
30 days	0.8±0.3	5.2±0.4	46.2±1.5
Mature (75 days)	6.4±0.9	12.7±0.4	68.8±1.4

*Figures are averages from at least two experiments.
†Not sex-differented; other age groups were female.

virtually absent in fetal liver but that it appears and increases with age. It is not known whether this increase is related to the hormonal status of the maturing animal. This phenomenon has already been described for DHA sulfate formation by Wengle (14), who used unfractionated liver supernatant preparations.

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In vitro biosynthesis of steroid sulfates by cell-free preparations from tissues of the laying hen[1]

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The sulfurylation of estrone-6,7-^{3}H, estradiol-17β-6,7-^{3}H, and dehydroisoandrosterone-4-^{14}C by laying hen liver, oviduct, and vaginal preparations was investigated. Purification and product identification included ether and ethyl acetate extraction, paper chromatography, and isotope dilution, before and after hydrolysis with a sulfatase preparation.

The esterifying enzymes were found in the 105 000 × *g* supernatants of the three tissues. The liver preparation was many times more active in steroid sulfate synthesis than the corresponding oviduct or vaginal fractions. Sulfurylation of dehydroisoandrosterone displayed the same cofactor, pH, and temperature requirements as did that of estrone and estradiol-17β. The degree of dehydroisoandrosterone sulfate synthesis was considerably lower than that of the estrogens, however. It is suggested that the laying hen possesses an enzyme system which is more efficient for the sulfurylation of estrogens than of other steroids such as dehydroisoandrosterone.

Introduction

The relegation of steroid sulfates purely to the position of detoxification products by early workers has recently been revised to bestow upon these compounds greater significance in the areas of biogenesis, metabolism, and possibly biological activity. Purdy, Engel, and Oncley (1) have indicated that estrone sulfate is a major circulating human estrogen. Since then the attention of investigators has been directed toward steroid conjugates, not only because they are excreted in that form, but owing to the demonstration that steroid sulfates may in some instances serve as biosynthetic intermediates (2–7). Little if any information related to the esterification of steroids, and specifically the sulfurylation of estrogens, by avian species has been provided, however. As a consequence, the present publication deals principally with the sulfate esterification of phenolic steroids by hen tissues.

Materials and Methods

Liver, oviduct, and vaginal tissue was obtained through the courtesy of Dr. N. Nikolaiczuk, of the Department of Animal Science, Macdonald College of McGill University, Montreal, from yearling White Rock hens in the laying state at the time of decapitation.

Adenosine triphosphate (ATP) was purchased from Sigma Chemical Company, St. Louis, Mo. Mylase P (containing sulfatase) is a product of Mann Research Laboratories Inc., New York, N.Y.

Pure crystalline estrone (3-hydroxy-estra-1,3,5(10)-trien-17-one), estradiol-17β (estra-1,3,5(10)-triene-3,17β-diol), and dehydroisoandrosterone (3β-hydroxy-Δ^5-androsten-17-one) were supplied by Mann Research Laboratories. Estrone-3-sulfate and dehydroisoandrosterone-3-sulfate were synthesized as described by Fieser (8). Estradiol-17β-3-sulfate was prepared by sodium borohydride reduction of estrone-3-sulfate. In the case of all three sulfate preparations, any free unreacted steroid was extracted from aqueous solution with diethyl ether, and the remaining butanol-soluble conjugated material crystallized several times from methanol – diethyl ether. Paper chromatography of the products in the solvent system ethyl acetate – *n*-butanol – 0.2% (v/v) NH_4OH (7:1:8) (9) showed homogeneous behavior in each case. Solutions of estrone-6,7-^{3}H (150 μCi/μg) and estradiol-17β-6,7-^{3}H (150 μCi/μg) were purchased from New England Nuclear Corp., Boston, Mass. Dehydroisoandrosterone-4-^{14}C (141 μCi/mg) was acquired from Merck, Sharp and Dohme, Montreal. When necessary, these were purified chromatographically and shown to be radiochemically pure by crystallization with added carrier. Crystallization was performed using methanol as solvent. Agreement, within the counting error, of specific activities of crystals and mother liquor was taken as proof of purity. All other chemical reagents were purified, where required, by standard procedures.

Measurement of Radioactivity

Radioactivity was measured by liquid-scintillation spectrometry in a Nuclear-Chicago model 6725 dual-channel instrument. The material to be assayed was dried in 20-ml glass scintillation vials, redissolved in the

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minimum volume of methanol, and this was diluted with 10 ml of toluene containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-1-benzene prior to counting. Sufficient counts were accumulated to ensure a counting error of less than ±5%. Quenching was estimated by the internal standard technique, and all counts were corrected to the efficiency of counting for ^{3}H and ^{14}C standards in 10 ml of the above scintillation fluid containing no methanol, namely, 26 and 63%, respectively.

Tissue Preparation, Incubation, Extraction, and Product Identification

Pooled livers, oviducts, and vaginae, excised from hens in the laying state, were fractionated by a method based on that of Schneider and Lewbart (9). Tissues were homogenized in a Virtis "45" homogenizer at 0 °C with 0.1 *M* phosphate buffer, pH 6.5, so that 10 ml of homogenate contained 2 g of original tissue. Nuclei and cell debris were sedimented at 1000 × *g* for 10 min. The supernatant fluids thus obtained were centrifuged at 105 000 × *g* for 45 min. The resulting 105 000 × *g* supernatants were stored in 2-ml portions at −20 °C and were used as the source of enzyme in all experiments. All centrifuging was performed in a Spinco model L preparative ultracentrifuge.

The procedure of incubation and extraction was schematically presented in a previous publication (10), but was slightly modified due to a different sub-cellular fraction being employed in the present study. The basic incubation system was of 3 ml total volume in all cases. It contained 0.013 *M* $MgCl_2$, 0.008 *M* K_2SO_4, 0.014 *M* ATP; estrone-6,7-^{3}H, estradiol-17β-6,7-^{3}H, or dehydroisoandrosterone-4-^{14}C (ca. 20 000 – 160 000 c.p.m.) diluted with the unlabelled form to give a final incubated concentration of 0.013 m*M*, the steroids being added in 0.1 ml propylene glycol; and 1 ml of the microsome-free enzyme preparation in 0.1 *M* phosphate buffer, pH 6.5 (equivalent to ca. 200 mg wet tissue) after pre-incubation for 10 min at 40 °C; the balance of the incubation volume consisted of distilled water. Incubation was performed in air at 40 °C for 2 h. Blank systems were identical to the above except that they contained no added ATP. Each of these experiments was performed in duplicate on one occasion and then later repeated in duplicate with tissue from other hens.

In the case of the liver preparation only, experiments were also performed in which ATP and substrate concentrations, as well as pH and temperature of incubation, were varied. The effect of varying ATP concentration over the range 0.0014–0.14 *M* was investigated while keeping the remainder of the basic incubation system (see above) unchanged. Likewise, in another experiment, substrate concentration was varied over the range 0.013 × 10^{-2} to 6.7 × 10^{-2} m*M* while otherwise preserving the basic system. In two further experiments, both using the basic incubation system, the pH of incubation was varied at 0.5-unit intervals from 6.0 to 8.0, and the effect of changing temperature was studied by incubating at 30, 37, 40, 42, and 48 °C.

Following all incubations, the reaction was arrested by the addition of excess methanol and the precipitate formed was centrifuged off. The latter was washed with methanol, which was combined with the first supernatant and evaporated *in vacuo*, with the addition of NH_4OH to prevent breakdown of steroid sulfates, to yield an aqueous residue. This latter was diluted with H_2O and extracted with diethyl ether in order to remove free steroids. The aqueous solution was then made 3 *M* with respect to NaCl and extracted with ethyl acetate to remove steroid sulfates. The ethyl acetate extract was dried over anhydrous Na_2SO_4 prior to evaporation *in vacuo* with the addition of NH_4OH. The residue was then dissolved in methanol, made alkaline with NH_4OH, and, if necessary, stored in the refrigerator until required. The amount of radioactivity in each extract was determined to give, in each experiment, a measure of "total steroid sulfate synthesized". The extracts were then further purified (sometimes following pooling of extracts from duplicate incubations) by methods described below, in order to identify, and to estimate the degree of purity of, the steroid sulfates formed.

In one experiment involving the basic incubation system and the liver preparation, pooled ethyl acetate (sulfate) fractions from duplicate incubations were subjected to descending paper chromatography as described by Schneider and Lewbart (9) in an ethyl acetate – *n*-butanol – 0.2% (v/v) NH_4OH (7:1:8) solvent system. The radioactivity corresponding to appropriate reference standards (estrone-3-sulfate, estradiol-17β-3-sulfate, or dehydroisoandrosterone-3-sulfate) was eluted with methanol. The methanol was removed under reduced pressure in the presence of NH_4OH, and the residue was stored in a vacuum desiccator over calcium chloride overnight in the refrigerator. The eluates obtained from the chromatograms were redissolved in methanol, and an aliquot (one-half) of each was submitted to successive crystallization, with the appropriate unlabelled pure crystalline steroid sulfate as carrier, in individual tared centrifuge tubes. Three crystallizations from methanol with the addition of a few drops of dry diethyl ether were carried out (11). The remaining one-half of the eluate was hydrolyzed with Mylase P (sulfatase) for 17 h at 37 °C, as described by Emerman *et al.* (12), and the resulting free (unconjugated) material was successively crystallized from methanol with pure unlabelled estrone, estradiol-17β, or dehydroisoandrosterone as carrier until equal and constant specific activity of crystals and mother liquor was attained.

The conjugated (ethyl acetate) extracts of one further set of experiments (in which estrone, estradiol-17β, and dehydroisoandrosterone had been incubated with the basic system using the liver preparation) were diluted directly with carrier estrone-3-sulfate, estradiol-17β-3-sulfate, and dehydroisoandrosterone-3-sulfate, respectively, followed by crystallization as described above. This was done in order to estimate how much of the radioactivity in these relatively crude extracts, prior to any chromatographic step, consisted of the expected steroid sulfates.

Direct crystallization following addition of carrier steroid sulfates to the ethyl acetate extracts was also performed in the experiments in which ATP and substrate concentration, and pH and temperature, were varied.

In the experiments involving tissue preparations of oviduct and vagina, the ethyl acetate extracts were incubated with Mylase P as described above, and the ether-soluble radioactivity so released was diluted with

TABLE I

Typical identification of the sulfurylated incubation products of estrone-6,7-^{3}H, estradiol-17β-6,7-^{3}H, and dehydroisoandrosterone-4-^{14}C in conjugated form*

Metabolite†					
Estrone-3-sulfate		Estradiol-17β-3-sulfate		Dehydroisoandrosterone-3-sulfate	
Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg
Paper chromatographic pool (81 820 c.p.m.)		Paper chromatographic pool (74 440 c.p.m.)		Paper chromatographic pool (3540 c.p.m.)	
+ 20 mg carrier	4091 (calcd.)	+ 20 mg carrier	3722 (calcd.)	+ 20 mg carrier	177 (calcd.)
Crystallization from methanol – diethyl ether		Crystallization from methanol – diethyl ether		Crystallization from methanol – diethyl ether	
Crystals 1	4026	Crystals 1	3726	Crystals 1	167
Mother liquor 1	4189	Mother liquor 1	3948	Mother liquor 1	189
Crystals 2	3858	Crystals 2	3439	Crystals 2	148
Mother liquor 2	3900	Mother liquor 2	3506	Mother liquor 2	155
Crystals 3	3840	Crystals 3	3450	Crystals 3	136
Mother liquor 3	3887	Mother liquor 3	3501	Mother liquor 3	146

*Products of liver metabolism only.
†Product directly identified by crystallization with pure unlabelled estrone-3-sulfate, estradiol-17β-3-sulfate, or dehydroisoandrosterone-3-sulfate following paper chromatography.

TABLE II

Typical identification of the sulfurylated incubation products of estrone-6,7-^{3}H, estradiol-17β-6,7-^{3}H, and dehydroisoandrosterone-4-^{14}C following Mylase P hydrolysis*

Metabolite†					
Estrone-3-sulfate		Estradiol-17β-3-sulfate		Dehydroisoandrosterone-3-sulfate	
Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg
Mylase P hydrolysate (80 120 c.p.m.)		Mylase P hydrolysate (74 860 c.p.m.)		Mylase P hydrolysate (2980 c.p.m.)	
+ 20 mg carrier	4006 (calcd.)	+ 20 mg carrier	3743 (calcd.)	+ 20 mg carrier	149 (calcd.)
Crystallization from methanol		Crystallization from methanol		Crystallization from methanol	
Crystals 1	3990	Crystals 1	3671	Crystals 1	144
Mother liquor 1	4092	Mother liquor 1	3900	Mother liquor 1	173
Crystals 2	3822	Crystals 2	3411	Crystals 2	130
Mother liquor 2	3886	Mother liquor 2	3459	Mother liquor 2	139
Crystals 3	3861	Crystals 3	3406	Crystals 3	134
Mother liquor 3	3916	Mother liquor 3	3455	Mother liquor 3	141

*Products of liver metabolism only.
†Product indirectly identified by crystallization with pure unlabelled estrone, estradiol-17β, or dehydroisoandrosterone following paper chromatography and Mylase P hydrolysis.

TABLE III

Crystallization of the sulfurylated incubation products of estrone-6,7-^{3}H, estradiol-17β-6,7-^{3}H, and dehydroisoandrosterone-4-^{14}C in conjugated form*

Metabolite†					
Estrone-3-sulfate		Estradiol-17β-3-sulfate		Dehydroisoandrosterone-3-sulfate	
Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg
Ethyl acetate extract (88 940 c.p.m.) + 20 mg carrier	4447 (calcd.)	Ethyl acetate extract (76 640 c.p.m.) + 20 mg carrier	3832 (calcd.)	Ethyl acetate extract (3240 c.p.m.) + 20 mg carrier	162 (calcd.)
Crystallization from methanol – diethyl ether		Crystallization from methanol – diethyl ether		Crystallization from methanol – diethyl ether	
Crystals 1	4301	Crystals 1	3609	Crystals 1	160
Mother liquor 1	4726	Mother liquor 1	3944	Mother liquor 1	188
Crystals 2	4182	Crystals 2	3640	Crystals 2	160
Mother liquor 2	4215	Mother liquor 2	3685	Mother liquor 2	169
Crystals 3	4131	Crystals 3	3624	Crystals 3	156
Mother liquor 3	4158	Mother liquor 3	3649	Mother liquor 3	164

*Products of liver metabolism only.
†Product directly identified by crystallization with pure unlabelled estrone-3-sulfate, estradiol-17β-3-sulfate, or dehydroisoandrosterone-3-sulfate without prior paper chromatography.

TABLE IV

Crystallization of the conjugated incubation products of estrone-6,7-^{3}H, estradiol-17β-6,7-^{3}H, and dehydroisoandrosterone-4-^{14}C following hydrolysis with Mylase P*

Metabolite†					
Estrone sulfate		Estradiol-17β sulfate		Dehydroisoandrosterone sulfate	
Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg
Mylase P hydrolysate (34 180 c.p.m.)		Mylase P hydrolysate (19 520 c.p.m.)		Mylase P hydrolysate (2120 c.p.m.)	
+ 20 mg carrier	1709 (calcd.)	+ 20 mg carrier	976 (calcd.)	+ 20 mg carrier	106 (calcd.)
Crystals 1	1669	Crystals 1	918	Crystals 1	98
Mother liquor 1	1800	Mother liquor 1	1009	Mother liquor 1	112
Crystals 2	1555	Crystals 2	881	Crystals 2	90
Mother liquor 2	1614	Mother liquor 2	917	Mother liquor 2	99
Crystals 3	1561	Crystals 3	873	Crystals 3	89
Mother liquor 3	1579	Mother liquor 3	860	Mother liquor 3	92

*Products of oviduct metabolism only.
†Product crystallized with pure unlabelled estrone, estradiol-17β, or dehydroisoandrosterone following Mylase P hydrolysis.

TABLE V

Crystallization of the conjugated incubation products of estrone-6,7-^{3}H, estradiol-17β-6,7-^{3}H, and dehydroisoandrosterone-4-^{14}C following hydrolysis with Mylase P*

Metabolite†					
Estrone sulfate		Estradiol-17β sulfate		Dehydroisoandrosterone sulfate	
Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg
Mylase P hydrolysate (6020 c.p.m.)		Mylase P hydrolysate (5020 c.p.m.)		Mylase P hydrolysate (220 c.p.m.)	—
+ 20 mg carrier	301 (calcd.)	+ 20 mg carrier	251 (calcd.)		
Crystals 1	281	Crystals 1	226		
Mother liquor 1	370	Mother liquor 1	306		
Crystals 2	226	Crystals 2	198		
Mother liquor 2	261	Mother liquor 2	228		
Crystals 3	230	Crystals 3	196		
Mother liquor 3	247	Mother liquor 3	204		

*Products of vaginal metabolism only.
†Product crystallized with pure unlabelled estrone or estradiol-17β; the dehydroisoandrosterone was not further processed.

the appropriate carrier steroid (estrone, estradiol-17β, or dehydroisoandrosterone) and crystallized.

Some measure of percentage sulfurylation in each experiment was obtained from the final specific activities of the crystallized material. The mathematical products of these values and the corresponding weights of carrier steroids, or steroid sulfates, yielded figures for radioactivity in the form of steroid sulfate formed on incubation. These latter values were then expressed as percentages of incubated radioactivity. It should be stressed that such a calculation does not take into account any losses occurring in the experiment prior to dilution with carrier steroid.

Results

Pooled ethyl acetate extracts from duplicate experiments, using the basic incubation conditions and the liver-enzyme preparation, were chromatographed on paper and the eluted zones corresponding to the individual steroid sulfates each divided into two parts. One half, crystallized with carrier steroid sulfates, yielded results shown in Table I. The other half, following sulfatase hydrolysis, and crystallized with carrier-free steroids, gave the results shown in Table II. It can be seen from Table I that 92–93% of the radioactivity eluted from the paper chromatograms as estrone-3-sulfate and estradiol-17β-3-sulfate behaved as these compounds during crystallization in the conjugated form. Moreover, Table II shows that the eluted radioactivity was for all practical purposes quantitatively hydrolyzed by Mylase P and that 91 and 96% of the free (unconjugated) radioactivity behaved as estradiol-17β and estrone, respectively, during crystallization. In the case of dehydroisoandrosterone-3-sulfate crystallized in the intact form (Table I), a greater relative decrease in specific activity was noted than for the two estrogen sulfates. Thus only about 77% of the radioactivity remained associated with the crystals. Also, it can be seen from Tables I and II that only about 84% of the conjugated radioactivity was freed by Mylase P and that 90% of this behaved like dehydroisoandrosterone on crystallization (Table II).

Table III contains data on the crystallization of the three steroid sulfates formed in another experiment using the liver-enzyme preparation under the basic experimental conditions. In this instance carrier steroid sulfates were added directly to the ethyl acetate (conjugated) extracts without any paper-chromatographic step. It can be seen that, although the first mother liquor in each case exhibited a higher specific activity than did the corresponding crystals, indicating the presence of an additional labelled component, the specific activities of the third crystals, when related to the initial calculated values, showed that about 93–96% of the radioactivity extracted with ethyl acetate behaved like the steroid sulfates under investigation.

Tables IV and V contain data on the purification of the conjugated material formed by oviduct and vaginal tissue preparations, respectively, under the basic incubation conditions. In the case of these products only partial characterization was attempted by crystallization of the free steroids following sulfatase hydrolysis. In every case more than 90% of the radioactivity in these ethyl acetate extracts was released by Mylase P hydrolysis. As can be seen from Tables IV and V, a major fraction of the radioactivity remained associated with the crystals although the counts were so low in the case of dehydroisoandrosterone from the experiment with vaginal tissue as to render the results meaningless. It appeared that there was a greater decrease in specific activity of estrone and estradiol-17β following hydrolysis in the case of the vaginal tissue preparation than for the oviduct.

Table VI contains data on the degree of steroid sulfate synthesis by the three tissue preparations under the basic experimental conditions. It should be pointed out that the values given for percentage substrate sulfurylated in the case of the liver system are minimal since the sulfates were subjected to a paper-chromatographic step during purification, resulting in some experimental loss of radioactivity. Percentage sulfurylation was calculated as follows, taking as an example the incubation of estradiol-17β-6,7-^{3}H (155 000 c.p.m.). Following incubation, ether-extractable (free) radioactivity amounted to 47 000 c.p.m. The ethyl acetate soluble (conjugated) material possessed most of the balance of the incubated radioactivity (about 78 000 c.p.m.), and 73 700 c.p.m. with a mobility similar to that of estradiol-17β-3-sulfate were eluted from the paper chromatogram. Crystallization with carrier indicated this to be 93% pure, giving a value of 68 500 c.p.m. for the estradiol sulfate formed, or 44.5% of the incubated radioactivity. The values for sulfurylation of the three steroids by oviduct and vaginal tissue preparations are based on crystallization data following hydrolysis of the 'sulfate-like' conjugates formed during incubation. It is obvious, nevertheless, that much less synthesis

TABLE VI

Steroid sulfate synthesis by enzyme preparations from laying hen liver, oviduct, and vagina*

Steroid substrate	Liver				Oviduct				Vagina			
	Free fraction, c.p.m. $\times 10^{-3}$	Sulfurylated fraction,† c.p.m. $\times 10^{-3}$	% Substrate sulfurylated‡	% Recovery of incubated activity	Free fraction, c.p.m. $\times 10^{-3}$	Sulfurylated fraction,† c.p.m. $\times 10^{-3}$	% Substrate sulfurylated‡	% Recovery of incubated activity	Free fraction, c.p.m. $\times 10^{-3}$	Sulfurylated fraction,† c.p.m. $\times 10^{-3}$	% Substrate sulfurylated‡	% Recovery of incubated activity
Estrone-6,7-^{3}H, 154 $\times 10^{3}$ c.p.m.	19.1	76.8	51.5±1.6	65±2.2	103	31.2	19.4±0.9	83±4.3	132	4.60	2.9±0.4	85±2.1
Estradiol-17β-6,7-^{3}H, 155 $\times 10^{3}$ c.p.m.	47.0	68.5	44.5±3.1	75±2.9	103	17.5	11.1±1.0	77±2.7	130	3.91	2.5±0.4	85±1.6
Dehydroisoandrosterone-4-^{14}C, 22 $\times 10^{3}$ c.p.m.	16.7	2.77	12.6±1.6	89±1.8	20.6	1.79	8.1±0.6	99±4.7	19.8	0.090	0.4±0.1	90±2.3

*Figures are averages of two experiments, each in duplicate. Substrate concn., 0.013 mM; ATP, 0.014 M; pH, 6.5; time, 2 h; temp., 40 °C.
†Values calculated following crystallization to constant specific activity; for oviduct and vagina only after hydrolysis.
‡All figures corrected for blank values, ranging between 1 and 3% of the sample value.

proceeded with these tissues than with the liver preparation. It is also clear from the values in Table VI that all three tissues systems were much more active, under the experimental conditions, in sulfurylating the two estrogens than was the case with dehydroisoandrosterone as substrate.

Attempts to increase sulfurylation of dehydroisoandrosterone relative to that of estrone and estradiol-17β by the liver system through variation of ATP concentration, pH, and temperature were not successful. It was found that maximum synthesis of all three steroid sulfates occurred at an ATP concentration between 0.007 and 0.014 *M*. Thus, the relative pattern was as shown in Table VI. Optimum sulfate synthesis for all three steroids also occurred at pH 6.5 and 40 °C, so that no combination of conditions employed resulted in any marked increase of dehydroisoandrosterone conjugation over that found using the basic incubation system described above. Also, dehydroisoandrosterone at a substrate concentration of 0.67×10^{-2} m*M* was sulfurylated to the extent of 4.0 mμmoles of material incubated. A 10-fold increase in concentration resulted in no further increase in sulfate synthesis. Estrone and estradiol-17β, on the other hand, had apparently not reached an absolute maximum in product formation, even at substrate concentrations as high as 6.7×10^{-2} m*M*, the weight of these two steroids sulfurylated being 42.8 and 33.6 mμmoles, respectively.

Discussion

It appears in the light of the present study that the 105 000 × *g* supernatant fraction of hen liver possesses a highly active enzyme system capable of sulfurylating estrone and estradiol-17β to form the corresponding 3-monosulfates. The experimental procedures employed for the extraction and purification of these products appear to have been successful in preserving the structure of the somewhat labile steroid sulfates, thus making it possible to crystallize them in their intact form as well as in the free form, following enzymatic hydrolysis. The activity of the liver system towards these two estrogens is considerably greater than that of oviduct or vaginal preparations, particularly since only partial identification can be claimed for the products in the case of the two latter tissues.

Dehydroisoandrosterone is also sulfurylated by the liver system, while a compound resembling dehydroisoandrosterone sulfate is formed by the oviduct preparation. Little, if any, such material was synthesized by the vaginal system. All three tissue preparations exhibited a much greater degree of sulfate conjugation of estrone and estradiol-17β than of dehydroisoandrosterone. Variation of incubation conditions in the case of the liver-enzyme system did not result in any increased sulfurylation of dehydroisoandrosterone as compared with that of the estrogens.

Earlier work in this laboratory (10) showed that an enzyme fraction prepared from a 20 000 × *g* supernatant of rat liver by precipitation between 1.7 and 2.3 *M* $(NH_4)_2SO_4$ was five to eleven times more active in sulfurylating dehydroisoandrosterone than estrone or estradiol-17β. Such salt-fractionated preparations of hen liver, oviduct, and vagina were inactive in our hands. Consideration of these findings suggests the possibility that structurally different proteins are operational in sulfurylation of steroids in varying species of test animal. Furthermore, since the laying hen is known to be continuously under the influence of circulating estrogens (13–16), it would seem logical that this species might possess an enzyme system which is more efficient in the sulfurylation of these phenolic steroids.

One further possible explanation for the low degree of sulfurylation found for dehydroisoandrosterone in the present work might relate to metabolism of this steroid during incubation to products incapable of being conjugated with sulfuric acid, or perhaps, to the further metabolism of synthesized dehydroisoandrosterone sulfate. This, however, is speculative at the present time and no attempt was made in this study to enquire into such a possibility.

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In vitro metabolism of estrone-4-^{14}C and estrone-6,7-^{3}H-3-sulfate by laying hen liver homogenates[1]

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Estrone-4-^{14}C and estrone-6,7-^{3}H-3-sulfate were simultaneously incubated with hen liver homogenates prepared from fowl in the laying stage. Purification and identification of conjugated metabolites was made by two different methods: (*a*) an indirect procedure involving hydrolysis with a sulfatase preparation, followed by ether extraction, Girard separation, Celite partition chromatography, and crystallization to constant specific activity of the resulting free estrogens; (*b*) a direct identification of estrogen sulfates by paper chromatography in three different solvent systems, again followed by recrystallization. The unconjugated (free) incubation products were identified by the former of the two methods.

Liver homogenates from the fowl were capable of catalyzing a rapid reduction of estrone to estradiol-17β and of estrone-3-sulfate to estradiol-17β-3-sulfate, the latter apparently without removal of the sulfate group. Under the conditions of the experiments, a slower dehydrogenation of estradiol-17β to estrone and of estradiol-17β-3-sulfate to estrone-3-sulfate was also indicated.

Introduction

Steroid sulfates have previously been shown to be of importance as intermediates in steroid interconversions (1–3). The majority of information related to the metabolism of estrogen sulfates has resulted from in vivo studies (4–8). With respect to phenolic steroid sulfate transformation, in vitro work has been almost nonexistent (9, 10), and has invariably involved species whose endogenous estrogen metabolic pattern is uncertain, and may, therefore, bear no relationship to the true conditions prevailing in the test animal.

One species, however, whose endogenous scheme of estrogen metabolism has been considerably elucidated is the fowl, the laying hen possessing a pattern qualitatively similar to that of the human (11–13). Consequently, it was considered conceivable that liver tissue from this source could metabolize estrogen sulfates, and accordingly such an investigation was undertaken.

Materials and Methods

Tissues and Chemicals

Pooled livers from two yearling White Rock hens in the laying period at the time of decapitation (obtained through the courtesy of Dr. N. Nikolaiczuk, of the Department of Animal Science, Macdonald College of McGill University, Montreal) were the source of the homogenate. Cofactors were acquired from Nutritional Biochemicals Corp., Cleveland, Ohio, except for adenosine triphosphate (ATP), which was purchased from Sigma Chemical Company, St. Louis, Mo. Mylase P (sulfatase) was obtained from Mann Research Laboratories Inc., New York, N.Y.

Estrone (3-hydroxy-estra-1,3,5(10)-trien-17-one) and estradiol-17β (estra-1,3,5(10)-triene-3,17β-diol), in pure crystalline form, were purchased from Mann Research Laboratories. Estrone-3-sulfate was synthesized as described by Fieser (14) and estradiol-17β-3-sulfate was prepared from it by reduction with sodium borohydride. Both compounds were purified by crystallization (15). Solutions of estrone-6,7-^{3}H (150 μCi/μg) and estrone-4-^{14}C (125 μCi/mg) were purchased from New England Nuclear Corp., Boston, Mass., and the Radiochemical Centre, Amersham, England, respectively. Estrone-6,7-^{3}H-3-sulfate was prepared from estrone-6,7-^{3}H by chlorosulfonic acid sulfurylation (14). The conjugated estrone so formed was extracted from aqueous solution, pH 10, with *n*-butanol; the extract was evaporated, dissolved in methanol, and chromatographed on paper in the solvent system toluene – *n*-butanol – NH_4OH – H_2O (5:5:1:9) (16). The area corresponding to the mobility of authentic estrone-3-sulfate was cut out and eluted with methanol. An aliquot of this, containing 77 400 c.p.m., was mixed with 22.3 mg of unlabelled estrone-3-sulfate, giving a calculated specific activity of 3471 c.p.m./mg, and crystallized from methanol – diethyl ether (17). Calculation of the specific activities of the crystals and mother liquor yielded values of 3492 and 3475 c.p.m./mg, respectively. Recrystallization resulted in values of 3479 and 3476 c.p.m./mg for crystals and mother liquor, respectively.

Celite No. 535, supplied by Johns–Manville and Company Ltd., was washed, and tested as described by Bauld and Greenway (18).

All other reagents were of suitable chemical grade, and

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were purified, where necessary, by previously published techniques (19).

The Radioactive-Tracer Technique

Measurement of radioactivity was performed by liquid-scintillation spectrometry in a Nuclear-Chicago model 6725 dual-channel instrument operating at an efficiency of 63 and 26% for ^{14}C and ^{3}H standards, respectively, in 10 ml of toluene containing 0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-1-benzene. Under these conditions, approximately 15% of the ^{14}C appeared in the ^{3}H channel, with about 1% of the ^{3}H counts spilling over into the ^{14}C channel. Materials to be counted were dried in 20-ml glass counting vials, dissolved in the minimum volume of methanol, and diluted with 10 ml of the above scintillation solution. Counts were corrected by the internal-standard technique, so that all values reported in this paper are for efficiencies of 63% for ^{14}C and 26% for ^{3}H. Calculation of ^{14}C and ^{3}H from doubly labelled samples was performed by the method of Okita *et al.* (20). The radioactivity of all samples was determined to within an error of at most ±5%.

Tissue Preparation, Incubation, Extraction, and Product Identification

Livers were excised, rinsed free of blood, weighed, and immediately used. The procedure of homogenate preparation, incubation, extraction, purification, and identification of metabolites has previously been described (21), and was adhered to in this study with a few notable exceptions.

The tissue was homogenized for 30 s in a Virtis 45 homogenizer in 0.1 *M* phosphate buffer, pH 7.4, containing 0.04 *M* nicotinamide. The ratio of buffer to wet weight of tissue was 1 ml/100 mg. In each incubation, 5 ml of the homogenate was used with the following added cofactors: NAD (2.3 μmoles), NADP (2 μmoles), glucose-6-phosphate (15 μmoles), ATP (10 μmoles), and $MgCl_2$ (279 μmoles). Estrone-4-^{14}C (6.0×10^5 c.p.m.) and estrone-6,7-^{3}H-3-sulfate (5.7×10^5 c.p.m.), diluted with their unlabelled forms giving 50 μg of each, were added to each incubation flask dissolved in 0.1 ml of propylene glycol. Incubations were carried out in duplicate for 5- and 90-min periods at 40 °C (21). Suitable blank incubations, utilizing the above components together with tissue homogenates which had been boiled for 20 min prior to incubation, were also carried out.

The incubations were terminated by the addition of excess acetone, the resulting protein precipitate being washed with acetone and discarded. Acetone was removed under reduced pressure and the free estrogen fraction extracted from the aqueous residue with diethyl ether. The estrone and estradiol-17β components of this were separated and purified, using the Girard reaction and partition column chromatography in the general manner described elsewhere (21). Radioactive estrone and estradiol-17β fractions were diluted with the appropriate unlabelled carrier steroids, and crystallized to constant specific activity and until the values for crystals and mother liquors agreed within the counting error. The mathematical product of the final specific activity of the crystals and the weight of carrier steroid added prior to crystallization then yielded some measure of the true radioactivity present in each metabolite. This was related to the incubated radioactivity to obtain percentage conversion.

The residual aqueous fraction from the above, following extraction of free steroids with ether, was adjusted to pH 12 with 1 *N* NaOH and extracted three times with an equal volume of water-saturated *n*-butanol to remove estrogen sulfates. The butanol was evaporated under reduced pressure while maintaining an alkaline pH; the residue was dissolved in methanol, and one half was chromatographed on Whatman No. 1 filter paper in each of three solvent systems. These were: ethyl acetate – *n*-butanol – 0.2% (v/v) conc. NH_4OH (7:1:8) (22); toluene – *n*-butanol – NH_4OH – H_2O (5:5:1:9) (16); toluene – *n*-butanol – 2.8% (v/v) conc. NH_4OH (3:1:2) (23). The running times varied between 4 and 7 h. Radioactivity of mobility corresponding to that of authentic estrone-3-sulfate or estradiol-17β-3-sulfate was eluted with methanol containing a small amount of NH_4OH. Each eluted fraction was diluted with unlabelled carrier estrone-3-sulfate or estradiol-17β-3-sulfate, whichever was applicable, and crystallized from methanol – diethyl ether (17). The final specific activities of the crystals were again used to obtain the total amount of radioactivity associated with the two estrogen sulfates, and this value was in turn related to the incubated radioactivity as a percentage. The remaining half of the butanol-soluble radioactivity was subjected to hydrolysis with the Mylase P enzyme preparation for 17 h at 37 °C (5), the freed material was extracted with diethyl ether, separated into fractions containing estrone and estradiol-17β by the Girard reaction, and purified by Celite partition column chromatography (19). Fractions with the properties of estrone or estradiol-17β were diluted with the appropriate carrier steroid and crystallized to constant specific activity.

Results

Following incubation, it was immediately obvious that considerable amounts of both ^{14}C- and ^{3}H-labelled materials were present in the free (ether-extractable) form. The butanol-soluble material, however, while containing significant amounts of ^{3}H originating from incubated estrone-3-sulfate, was practically devoid of ^{14}C which would originate from incubated free estrone. Thus in two experiments at 5 and 90 min, respectively, only 7971 and 8528 c.p.m. of ^{14}C were found in the butanol fraction. These values correspond to only 1.3 and 1.4% of incubated activity and are even less significant when it is considered that the blank incubation yielded 5613 c.p.m. in this 'conjugated' fraction. Since these small amounts of radioactivity were not rendered ether-soluble by Mylase P treatment, they were not further processed.

Table I shows crystallization data for the ^{3}H-labelled butanol-soluble metabolites (from one half of pooled butanol extracts of duplicate experiments) in the conjugated form following

TABLE I

Typical identification of incubation products of estrone-6,7-^{3}H-3-sulfate in conjugated form*

Metabolite†			
Estrone-3-sulfate		Estradiol-17β-3-sulfate	
Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg
Paper chromatographic pool (4420 c.p.m.) + 20 mg carrier	221 (calcd.)	Paper chromatographic pool (38 140 c.p.m.) + 20 mg carrier	1907 (calcd.)
Crystallized from methanol – diethyl ether		Crystallized from methanol – diethyl ether	
Crystals 1	206	Crystals 1	1467
Mother liquor 1	240	Mother liquor 1	1507
Crystals 2	192	Crystals 2	1439
Mother liquor 2	199	Mother liquor 2	1450
Crystals 3	190	Crystals 3	1441
Mother liquor 3	195	Mother liquor 3	1425

*After a 5-min incubation period.
†Product directly identified by crystallization with pure unlabelled estrone-3-sulfate or estradiol-17β-3-sulfate following paper chromatography.

TABLE II

Typical identification of incubation products of estrone-6,7-^{3}H-3-sulfate in free form following Mylase P hydrolysis of the conjugate fraction*

Metabolite†			
Estrone-3-sulfate		Estradiol-17β-3-sulfate	
Purification step	Specific activity, c.p.m./mg	Purification step	Specific activity, c.p.m./mg
Celite column pool (4140 c.p.m.) + 20 mg carrier	207 (calcd.)	Celite column pool (36 880 c.p.m.) + 20 mg carrier	1844 (calcd.)
Crystallized from methanol		Crystallized from methanol	
Crystals 1	197	Crystals 1	1441
Mother liquor 1	216	Mother liquor 1	1516
Crystals 2	180	Crystals 2	1418
Mother liquor 2	190	Mother liquor 2	1431
Crystals 3	182	Crystals 3	1414
Mother liquor 3	190	Mother liquor 3	‡

*After a 5-min incubation period.
†Product indirectly identified by crystallization with pure unlabelled estrone or estradiol-17β following Mylase P hydrolysis and column chromatography.
‡Lost.

paper chromatography. After three crystallizations, about 86% of the radioactivity, behaving like estrone-3-sulfate on paper, remained associated with the carrier while 76% of the presumptive estradiol-17β-3-sulfate appeared to be in the form of that compound. The other half of the conjugate fraction from these same incubations, following hydrolysis with the Mylase P preparation and purification by the Girard reaction and partition column chromatography, was then identified as free steroid by crystallization with carrier. Table II shows the results of such crystallizations, and it can be seen that about 88% of the radioactivity eluted from the column in the position of free estrone remained associated with the crystals after three crystallizations. The corresponding value for estradiol-17β was 77%. Table III shows data for the degree of conversion of estrone-4-^{14}C and estrone-6,7-^{3}H-3-sulfate by the liver homogenates. It should be noted that in these experiments only estrone and its sulfate and estradiol-17β and its sulfate were sought and investigated. Percentage conversion was calculated as in the following example. In the 5-min

TABLE III

The metabolism of estrone-4-^{14}C and estrone-6,7-^{3}H-3-sulfate by hen liver homogenate in vitro*

Steroid substrate	Time of incubation, min	Activity in estrone fraction,† c.p.m. $\times 10^{-3}$	Activity in estradiol-17β fraction,† c.p.m. $\times 10^{-3}$	Activity in estrone-3-sulfate fraction,† c.p.m. $\times 10^{-3}$	Activity in estradiol-17β-3-sulfate fraction,† c.p.m. $\times 10^{-3}$	% Conversion to: Estrone	Estradiol-17β	Estrone-3-sulfate	Estradiol-17β-3-sulfate
		^{14}C	^{14}C	^{14}C	^{14}C				
Estrone-4-^{14}C	5	120	68.1	0	0	20.0±1.0	11.4±0.9	0	0
	90	160	41.8	0	0	26.7±1.4	7.0±0.2	0	0
	Blank (boiled tissue)	244	2.38	0	0	40.8±0.6	0.4±0.2	0	0
		^{3}H	^{3}H	^{3}H	^{3}H				
Estrone-6,7-^{3}H-3-sulfate	5	80.6	44.2	3.72	28.6	14.1±1.0	7.7±0.6	0.7±0.2	5.0±0.4
	90	98.2	28.6	26.7	10.5	17.1±1.4	5.0±0.9	4.7±0.4	1.8±0.4
	Blank (boiled tissue)	37.4	3.62	116	0.870	6.5±1.0	0.6±0.2	20.2±0.9	0.2±0.0

*Figures are averages of two experiments, each in duplicate.
†Values calculated following crystallization to constant specific activity.

experiment, when 5.7×10^5 c.p.m. of estrone-6,7-^{3}H-3-sulfate were incubated, 4325 c.p.m. of 'estrone sulfate' and 37 600 c.p.m. of 'estradiol-17β sulfate' were eluted from paper chromatograms of the type described above. Following crystallization with added carriers, in the conjugated form, 3700 c.p.m. remained associated with the estrone-3-sulfate carrier and 28 500 c.p.m. with the estradiol-17β-3-sulfate carrier. These corresponded to 0.65 and 5.0%, respectively, of the incubated substrate. It should be noted that such a calculation does not take into account experimental losses prior to the point of addition of carrier steroid, thus the values given in Table III are minimal in nature. It is apparent from the data in Table III that interconversion of estrone and estradiol-17β, both in the free and sulfate forms, occurred.

Discussion

It is immediately apparent from the results in this paper that free estrone may be metabolized to estradiol-17β very rapidly by the liver homogenate. In the first 5 min of incubation, 11.4% of the estrone was transformed to the dihydroxy compound. After a 90-min incubation period this conversion had decreased to an apparent value of 7.0% with a concomitant increase in estrone from 20.0% at 5 min of incubation to 26.7% after 90 min. The data suggest that the estrone to estradiol-17β conversion by hen liver tissue is a readily reversible reaction, with the estrone to estradiol-17β transformation occurring almost immediately after exposure of substrate to tissue. In addition, other studies involving the simultaneous incubation of equal weights of estrone-4-^{14}C and estradiol-17β-6,7-^{3}H by this tissue have demonstrated that the reduction of estrone to estradiol-17β is considerably more rapid than the opposite oxidation reaction (24). However, it must be emphasized that such a situation may well be merely a reflection of the availability of cofactors in the in vitro system studied. Since the interconversion of estrone and estradiol-17β in free or sulfurylated form was of primary interest, the metabolism of either estrone or estrone-3-sulfate to other products is not reported upon in this paper.

Perhaps the most interesting finding in the present study concerns the metabolism of estrone-3-sulfate by the liver preparation. Table III indicates that the radioactive materials identified as estrone-3-sulfate and estradiol-17β-3-sulfate were exclusively ^{3}H-labelled with no ^{14}C detected in either fraction. Since the ^{3}H originates from the incubated estrone-6,7-^{3}H-3-sulfate while the corresponding free estrone was ^{14}C-labelled in the incubation mixture, this would tend to indicate a lack of sulfurylation of the free estrone-4-^{14}C by the whole liver homogenate. This latter finding may be due to the presence of inhibitors of the sulfurylation process, or perhaps to a low concentration of sulfokinase or active sulfate synthesizing systems, especially since microsome-free preparations of hen liver were highly active in this respect (15). Whatever the reason, this lack of esterification in combination with a 5.0% conversion of estrone-6,7-^{3}H-3-sulfate to estradiol-17β-6,7-^{3}H-sulfate after only 5 min of incubation (Table III) was strongly suggestive of a direct reduction without prior hydrolysis of the sulfate moiety. After 90 min of incubation, the level of this radioactive estradiol-17β-3-sulfate had diminished to 1.8% of the incubated estrone-3-sulfate, but was accompanied by an increase of labelled estrone-3-sulfate from 0.7% after a 5-min incubation period to 4.7% at 90 min. It is tempting to suggest that such data indicate an initial rapid reduction of estrone-3-sulfate to estradiol-17β-3-sulfate followed by a slower reverse reaction in much the same manner as described above for these two compounds in the free form. However, the recovery of the sulfated metabolites is very low (although fairly consistent), making such a claim a difficult one to defend with the information available. Nevertheless, the proof of identity of the steroids, both in the free and conjugated forms, is good enough to show the presence of enzymes (or an enzyme) in the whole homogenate capable of catalyzing, with added cofactors, some degree of interconversion between estrone and estradiol-17β on the one hand and estrone-3-sulfate and estradiol-17β-3-sulfate on the other.

It is noted from Table III that estrone-6,7-^{3}H-3-sulfate also gave rise to large amounts of free estrone and estradiol-17β. Thus, 5 min of incubation yielded 14.1% estrone-6,7-^{3}H and 7.7% estradiol-17β-6,7-^{3}H. The corresponding figures for 90 min were 17.1 and 5.0%, respectively. By observing the high blank value for free estrone production (6.5%), one is forced to conclude that the hydrolysis of the substrate estrone-6,7-^{3}H-3-sulfate was a result of both enzymatic and

non-enzymatic activity, the latter being the result of the lability of estrogen sulfates in general.

Finally, it must be emphasized that although the total recoveries of metabolites reported in Table III appear low, only the values for estrone and estradiol-17β in free or conjugated form were reported, with figures for other metabolites of estrone or estrone-3-sulfate being omitted. It must also be pointed out that no correction has been made for losses occurring during extraction of tissue, purification, etc.

In conclusion, evidence has been presented establishing the pattern of estrone and estradiol-17β interconversion by hen liver to be a readily reversible situation, with the estrone to estradiol-17β transformation taking place almost instantaneously. Furthermore, the respective C-3 ester sulfates of these compounds can exhibit a qualitatively similar behavior without prior hydrolysis of the sulfate moiety. It should be noted that all incubations were performed in phosphate buffer, and since phosphate ion is known to inhibit enzymic hydrolysis of steroid sulfates, the results obtained may merely reflect the activity of a suitable in vitro system. However, this does not alter the finding that the tissue investigated appears to possess the activity necessary to interconvert estrone-3-sulfate and estradiol-17β-3-sulfate. To the knowledge of the authors, the only other published works which have demonstrated the capability of liver tissue to transform estrogen sulfates in vitro without hydrolysis of the ester grouping are those of Crepy and Jayle (9) and Dahm and Breuer (10). They utilized tissues from the rabbit and rat, however.

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METABOLISM OF STEROID ESTROGENS IN THE HEN

IV. CONVERSION IN VIVO OF ESTRADIOL-17β-6,7-^{3}H-3,17-DISULFATE TO ESTRADIOL-17β-6,7-^{3}H-17-SULFATE.*

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ABSTRACT

Estradiol-17β-6,7-^{3}H-3,17-disulfate was injected intramuscularly into a mature non-laying hen. Very little (approximately 2%) of the injected material was recovered unchanged from the urine. The only radioactive metabolite identified in the urine was estradiol-17β-17-sulfate. The identification of this compound is based on: (a) Mylase P enzyme incubations; (b) methylation followed by solvolysis and thin-layer chromatographic identification of estradiol-17β-3-methyl ether; (c) acetylation of the latter and t.l.c. identification of estradiol-17β-3-methyl ether-17-acetate; (d) crystallization with unlabelled estradiol-17β-17-sulfate; (e) solvolysis of the latter and crystallization with unlabelled estradiol-17β.

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INTRODUCTION

The hen is known to excrete steroid estrogens in urine mainly, if not entirely, in conjugation with sulfuric acid (1). In a previous study (2), it was found that this species is capable of metabolising estrone sulfate to monosulfates and disulfates of the two estradiol epimers without prior hydrolysis of the sulfate group; hence the disulfates of the two estradiol epimers were presumably formed by sulfation of the C-17 hydroxyl group on each of the estradiol-3-sulfate molecules.

The present study was undertaken to investigate the fate of injected estradiol-17β-3, 17-disulfate and to establish whether or not the conversion of estradiol-3-sulfate to its disulfate form is a reversible process in vivo.

EXPERIMENTAL

Materials and Methods.

The experimental bird was a mature non-laying Rhode Island Red hen modified surgically (3) so as to exteriorize the ureteral openings and permit collection of the urine. Approximately 10 ml of 1 N NaOH solution was added to the collection tube each day in order to maintain an alkaline pH. This was done to avoid any possible breakdown of the urinary estrogen sulfates during collection of urine. The pH values of the 24-hr urine collections on each of the five days were found to lie between 8 and 9. The urine was removed from the collection tubes several times daily and placed immediately in cold storage at -15°C. The urine samples were processed immediately after each 24-hr collection was completed.

Estradiol-17β-6, 7-^{3}H-3, 17-disulfate was synthesized from estradiol-17β-6, 7-^{3}H (specific activity 147 μc/μg). This latter was purchased from New England Nuclear Corp., Boston, Mass. and shown to be at least 98% pure by crystallization with unlabelled

carrier. Sulfurylation was performed by the method of Fieser (4), unreacted steroid was extracted with ether and the conjugate was extracted with n-butanol from an aqueous solution of pH10. The latter material was chromatographed on a 40 g column of Woelm, neutral, activity grade I, aluminum oxide in methanol with an increasing concentration of NH_4OH. The radioactivity was quantitatively eluted as a sharp peak with methanol containing 0.15% (v/v) conc. NH_4OH. This labelled material was then applied in aqueous solution to a DEAE-Sephadex column (0.9 x 50 cm), which was then eluted with a linear gradient of zero to 0.8 M NaCl. Approximately 1.5% of the applied radioactivity was eluted at about 0.4 M NaCl, which is characteristic of a monosulfate conjugate (unpublished observation). No further radioactivity was eluted up to 0.8 M NaCl but continued elution with a second linear gradient of 0.8 - 2.0 M NaCl led to the appearance of a symmetrical peak at about 1.1 M NaCl. This accounted for 96% of the chromatographed material and it was recovered by treatment with Amberlite XAD-2 as described by Bradlow (5). Chromatography on Celite in the system iso-octane: t-butanol: M NH_4OH (2:5:5 v/v) (6) resulted in less than 1% of the radioactivity being recovered in the form of free steroid or monosulfate. The presumptive disulfate was eluted at about column volume 9. This latter material was not recovered from unacidified 3 M NaCl by ethyl acetate extraction, in contrast to the behaviour of monosulfates (7). Incubation of the disulfate with Mylase P (8) released no ether-soluble radioactivity but enabled quantitative partition of the radioactivity into ethyl acetate from 3 M NaCl suggesting the production of (presumably) the 17-monosulfate. The latter was completely solvolyzed in ethyl acetate-H_2SO_4 to yield ether-soluble material. The disulfate (480,000 dpm) was mixed with 28.1 mg of authentic unlabelled carrier (calculated specific activity 17,100 dpm/mg). Two successive crystallizations from methanol-ether yielded crystals of specific activity 18,300 and 18,900 dpm/mg, respectively. A further 113,400 dpm of the disulfate were solvolyzed, mixed with 18.1 mg of crystalline estradiol-17β (calculated specific activity 6270 dpm/mg) and crystallized twice as above. Crystals of specific activity 6170 and 6200 dpm/mg were obtained.

Reference steroid estrogens were purchased from Steraloids, Inc., Pawling, N.Y., U.S.A. or Mann Research Labs., New York, N.Y. Reference estrone-3-sulfate was a gift from Dr. D.S.Layne, Department of Biochemistry, University of Ottawa. Reference estradiol-17β-3-sulfate and reference estradiol-17β-3,17-disulfate were gifts from Dr. R.Y.Kirdani, Roswell Park Memorial Institute, Buffalo, N.Y.

Estradiol-17β-17-sulfate was prepared by incubation of estradiol-17β-3,17-disulfate with Mylase P (Mann Research Labs., New York, N.Y.) in 0.1 M acetate buffer, pH 6. It was re-crystallized from methanol: diethyl ether.

Amberlite XAD-2 synthetic ion exchange resin was purchased from Rohm and Haas, Philadelphia, Pa., U.S.A. Florisil (100-200 mesh) was purchased from Fisher Scientific Co., New Jersey, U.S.A. DEAE-Sephadex, A-25, was purchased from Pharmacia (Canada) Ltd., Montreal, Que.

All solvents, except methanol, were distilled before use. The methanol was 'spectranalysed' grade, Fisher Scientific Co., Montreal. Diethyl ether was purified as described by Bauld (9).

Thin-layer chromatography (t.l.c.) was performed on Silica Gel G (Merck). The solvent systems used are listed in Table I. Visualization of estrogens and of their derivatives was done as described elsewhere (2).

TABLE I

Composition of chromatographic systems used.

System	Composition by volume	Reference
1	Benzene-ethyl methyl ketone-ethanol-water (3:3:3:1)	10
2	2-Propanol-chloroform-methanol-10 N ammonia (10:10:5:2)	10
3	Chloroform-ethanol (3:1)	11
4	Cyclohexane: ethyl acetate (1:1)	12
5	n-Hexane: ethyl acetate (3:1)	2
6	Chloroform: diethyl ether (6:4)	13

Radioactivity was assayed (14) and acetylations were performed (15) as described earlier. Methylations were done by the method of Brown (16).

Experimental procedure.

Estradiol-17β-6, 7-^{3}H-3, 17-disulfate (14.6×10^{6} dpm) was dissolved in 0.5 ml of dimethyl sulfoxide and injected intramuscularly into the experimental hen. Urine was collected over five successive 24-hr periods. Each 24-hr urine sample was separately filtered and assayed for radioactivity.

Extraction of urinary steroids.

The urine from day-1 was passed through a column of Amberlite XAD-2. Steroids and steroid sulfates were eluted from the column with methanol as described by Bradlow (5). Two other frac-

tions, viz., (a) filtered urine and (b) water-wash of the XAD-2 column prior to its elution with methanol, were also obtained. Each of these three fractions was assayed for radioactivity.

Aliquots of the fractions containing the filtered urine and the water-wash of the column were hydrolysed separately by adding 15 volumes conc. HCl per 100 volumes and then boiling the mixture for 1 hour under reflux. The hydrolysed solutions were extracted with diethyl ether (3x1 vol) to remove any free estrogens. The ethereal extracts were assayed separately for radioactivity.

Fractionation of urinary steroid extract.

The methanol eluate obtained from the XAD-2 column was further fractionated on a Florisil column to yield (a) "free" estrogens, (b) monosulfates of estrogens and (c) disulfates of estrogens as described elsewhere (1).

Disulfate Fraction.

Lack of sufficient radioactivity in the presumptive disulfate fraction precluded rigorous identification. This fraction was chromatographed, however, in admixture with reference estradiol-17β-3, 17-disulfate in systems 1 and 2 and histograms of the chromatoplates were prepared.

Monosulfate Fraction.

An aliquot of the monosulfate fraction was chromatographed (t.l.c.) in system 1 along with reference estrone sulfate and estradiol-17β-3-sulfate. Fractions containing estrone-3-sulfate and estradiol-17β-"monosulfate" were obtained from the chromatoplate as described elsewhere (2). The fractions thus obtained were designated (a) presumptive estrone sulfate fraction and (b) presumptive estradiol "monosulfate" fraction. Aliquots of each fraction were assayed for radioactivity. The presumptive estradiol "monosulfate" fraction was chromatographed (t.l.c.) in systems 1, 2 and 3 along with reference estradiol-17β-3-sulfate.

The following procedural steps were taken in order to establish the identity of the presumptive estradiol-"monosulfate." An aliquot of the estradiol "monosulfate" fraction was incubated in 25 ml of acetate buffer, pH 6.0, with Mylase P enzyme preparation. A blank incubation containing no Mylase P was performed simultaneously. The incubations, in duplicate, were carried on for 24-hr at 37°C. The incubated solutions were thereafter extracted separately with chloroform (3x1 vol.) in order to remove any free estrogens which might have been liberated as a result of Mylase P activity.

A fresh aliquot of the total monosulfate fraction was methylated. The methylation product was adjusted to pH 10 with 5 N NaOH

and extracted in n-butanol, the n-butanol was evaporated under reduced pressure and the residue was dissolved in 1 ml of methanol. To this was gradually added, with shaking to mix thoroughly, 200 ml of ethyl acetate and the methyl ether(s) of the monosulfate fraction were subjected to solvolysis for 48 hrs. (1). The solvolysed material, after being washed with 1 M $NaHCO_3$ and H_2O (2), was evaporated to dryness under reduced pressure. The dry residue was chromatographed (t.l.c.) in systems 4 and 6, along with reference estradiol-17β-3-methyl ether. Histograms of the developed chromatoplates were made and the position of the peak of radioactivity observed was compared with that of the stain due to reference estradiol-17β-3-methyl ether.

A further aliquot of the solvolysed methylated product was acetylated and the acetylation product was chromatographed in system 5 in admixture with reference estradiol-17β-3-methyl ether-17-acetate. A histogram of the chromatoplate was made and the position of the peak of radioactivity was compared as to chromatographic mobility with the stain due to the reference estradiol-17β-3-methyl ether-17-acetate.

Yet a further portion of the total monosulfate fraction was crystallised with reference estradiol-17β-17-sulfate.

A fresh aliquot of the total monosulfate fraction was solvolysed for 48 hr. The solvolysed material was washed with 1 M $NaHCO_3$ and H_2O and the organic phase (ethyl acetate) was evaporated to dryness. The dry residue was chromatographed in system 6 and zones corresponding in chromatographic mobility with reference estrone, estradiol-17α, estradiol-17β and 16-epiestriol were removed from the chromatoplate, eluted with methanol and each fraction was assayed for radioactivity. The fraction containing estradiol-17β thus obtained was crystallised to constant specific activity with reference estradiol-17β.

RESULTS

The recovery of administered radioactivity from the urine from the first day was 8.0%. The total recovery for days 1-5 was 11.1%.

The recovery of the radioactivity from the Amberlite XAD-2 column (see Table II) was almost 100%. The steroid-containing methanol eluate from the Amberlite XAD-2 column accounted for only 40% of the total radioactivity. Acid hydrolysis (by 15% vol HCl conc.)

of the filtered urine fraction and of the water-wash, followed by ether extraction, did not yield any radioactivity in the ether extract, and 100% of the radioactivity was found in the aqueous phase in both cases.

TABLE II

Elution pattern of day-1 urine from Amberlite XAD-2 column. Method as described by Bradlow (5)

Fraction	Radioactivity (^{3}H)	
	dpm x 10^{-4}	Percent of total
1. Filtered urine	22.0	18.3
2. Water-wash	49.8	41.5
3. Methanol eluate	48.0	40.2
Total	119.8	100.0
Percentage of total urinary activity recovered from the column		100

Elution of the steroid fraction from a Florisil column with an increasing concentration of methanol in $CHCl_3$ was found to yield a pattern almost identical with the one obtained in previous studies (1, 2). The monoconjugate fraction accounted for over 95% of the total radioactivity whereas the diconjugates accounted for only about 2% of the total radioactivity. (Table III). The latter material had the chromatographic mobility of standard estradiol-17β-3, 17-disulfate in each of two t.l.c. systems.

The estrone sulfate fraction obtained from the presumptive monosulfate fraction by t.l.c. exhibited practically no radioactivity, which suggested absence of estrone. Practically all the radioactivity was found to be in the estradiol "monosulfate" fraction, the chromato-

graphic mobility of which corresponded to that of reference estradiol-17β-3-sulfate in three t.l.c. systems.

TABLE III

Florisil Column: Elution of the total steroid fraction of hens' urine and results of thin layer chromatography of resulting sub-fractions. Method used is as described elsewhere (1).

Fraction	Eluent	Radioactivity dpm x 10^{-3}	Radioactivity Percent of total	Identification by t.l.c.
1	$CHCl_3$	-	-	-
2	$CHCl_3$:MeOH 80:20	3.20	0.7	Free estrogen
3	" " 70:30	129	27.8	Mainly single conjugate + some free estrogens
4	" " 55:45	320	68.9	Single conjugate
5	" " 40:60	7.29	1.6	Mixture of two conjugates
6	" " 75:25	3.66	0.8	Double conjugate
7	Methanol	1.14	0.2	" "
	Total	464	100.0	
	Percentage of total radioactivity recovered from Florisil column		96.5%	

TABLE IV

Radioactivity extracted by chloroform after incubation of the presumptive estradiol "monosulfate" fraction of urinary estrogens. Incubations done for 24-hr at 37°C in 0.1 M acetate buffer, pH 6.0.

Enzyme treatment	A dpm in aliquot incubated (x 10^{-2})	B dpm in $CHCl_3$ extract (x 10^{-2})	$\frac{B}{A}$ x 100
1. Blank	773	24.3	3.1
2. Mylase P	773	28.6	3.7
3. Blank	247	14.8	6.0
4. Mylase P	247	21.7	8.8

Incubation of estradiol monosulfate fraction with Mylase P did not result in any increased amount of chloroform-extractable radioactivity over the corresponding blank used. This suggests that the sulfate group of the estradiol monosulfate fraction was not at the C-3 position but rather at C-17 of estradiol-17β. The results from the Mylase P incubations are presented in Table IV.

Further identification of the presumptive monosulfate fraction.

Methylation of the presumptive monosulfate fraction, followed by solvolysis and subsequent development on a thin-layer chromatoplate yielded one peak of radioactivity which corresponded in chromatographic mobility with the reference estradiol-17β-3-methyl ether in systems 4 and 6. This suggested that the urinary extract after the aforesaid manipulations yielded estradiol-17β-3-methyl ether. This was to be expected if the original material was estradiol-17β-17 sulfate.

Acetylation of the methyl ether and subsequent chromatography in system 5 once again yielded one peak of radioactivity which corresponded in chromatographic mobility with reference estradiol-17β-3-methyl ether-17-acetate.

These observations suggested strongly that the presumptive monosulfate fraction was almost entirely estradiol-17β-17-sulfate. Crystallization of the presumptive estradiol-17β-17-sulfate with unlabelled estradiol-17β-17 sulfate yielded the results presented in Table V, which confirmed the foregoing supposition.

TABLE V

Presumptive estradiol-17β-17 sulfate fraction:- Crystallization with reference estradiol-17β-17 sulfate.

	dpm x 10^{-3}	mg	$\frac{\text{dpm x } 10^{-1}}{\text{mg}}$
Original Pool	41.3	20.50	201
1st Crystallization Crystals	33.5	15.25	220
2nd Crystallization Crystals	29.7	13.50	220

Solvolysis of the monosulfate fraction and subsequent chromatography on thin-layer chromatoplates in system 6 revealed the following distribution of radioactivity on the chromatoplate:-

3.2% in the 16-epiestriol fraction; 82.7% in the estradiol-17β fraction; nil in the estradiol-17α fraction; and 1.6% in the estrone fraction.

These values suggested the virtual absence of estrone and of estradiol-17α and the presence of a high proportion of estradiol-17β. The 16-epiestriol fraction was not processed due to lack of sufficient radioactivity. The radioactive estradiol-17β fraction was crystallized to constant specific activity with reference estradiol-17β. The results, which are presented in Table VI, confirmed the chromatographic identification of the estradiol-17β.

TABLE VI

Presumptive estradiol-17β-17 sulfate: Identification of estradiol-17β following solvolysis of monosulfate fraction.

	dpm x 10^{-3}	mg	$\frac{\text{dpm x } 10^{-1}}{\text{mg}}$
Original Pool	114	43.50	262
1st Crystallization Crystals	95.2	35.91	265
2nd Crystallization Crystals	69.1	26.13	264

DISCUSSION

The recovery of tritium activity (11%) in the urine in the experiment now reported may be compared with the recovery of 16.6% of the radioactivity of injected estrone sulfate (2) and of 31% of the radioactivity of injected estradiol-17β (1) in other experiments. This comparison might suggest that the recovery in the urine decreases as the degree of sulfation of the injected steroid estrogen increases. A satisfactory resolution of this problem is unlikely, however, until more is known about the transfer of estrogens and estrogen conjugates through the bile.

In the present work only 41% of the total urinary radioactivity was recovered in the total urinary steroid ('free' + conjugated) fraction as compared with values of 97% and 93% when estradiol-17β (1) and estrone sulfate (2), respectively, were injected. This suggested that a relatively high proportion of injected estradiol-17β-3, 17-

disulfate was converted to highly polar compounds; and this suggestion was reinforced by the observation that acidic hydrolysis of the urine fraction which had been filtered through the Amberlite XAD-2 column did not liberate any appreciable amount of radioactive material extractable by diethyl ether. The radioactivity in that fraction, therefore, could not have been associated with any of the better-known steroid estrogens. Similar results were observed with aliquots of the water-wash of the XAD column despite the fact that this fraction accounted for 42% of the radioactivity passed through the column.

The partially purified estradiol "monosulfate" fraction, when incubated with the enzyme preparation Mylase P, did not release any radioactivity which could be extracted with chloroform. This observation, together with the behaviour of the monosulfate fraction on t.l.c., showed that the injected estradiol-17β-3, 17-disulfate had, in fact, given rise to estradiol-17β-17-sulfate. There can be little doubt as to the identity of the main component of the monosulfate fraction, the identification being based on the formation of well-defined derivatives, crystallization with reference estradiol-17β-17 sulfate and with reference estradiol-17β following cleavage of the sulfate group by solvolysis.

Mono- and disulfates of estradiols have been obtained from urine following injection of estrone sulfate labelled with ^{3}H and ^{35}S (2). The results now obtained indicate clearly that, at least as reflected in urinary metabolites, the formation of estradiol-17β-3, 17-

disulfate from estradiol-17β-3-sulfate in the hen is not a reversible process. Instead the injected disulfate of estradiol-17β loses the sulfate group from its C-3 position to yield estradiol-17β-17-sulfate. In the previous experiment (2), the sulfate group at the C-3 position remained attached to the estrogen molecule when estrone sulfate was converted to the 3-sulfates of the two estradiol epimers and finally to their disulfates.

In other experiments (17) the relative proportions of radioactive estradiol-17β to radioactive estrone in the urine of a non-laying hen following intramuscular injection of estrone-4-^{14}C or estradiol-17β-4-^{14}C were approximately 5: 1. If free estradiol-17β had been formed from the injected estradiol-17β-disulfate in the present experiment, one would expect radioactive estrone to have been present in the urine, unless estradiol-17β in this experiment was formed and reconjugated in a compartment lacking estradiol-17β dehydrogenase activity. In point of fact, no radioactive estrone was detected in the urine despite attempts to find it.

In previous studies (1, 2) the amounts of estrogen disulfates in hens urine have been found to account for only about 5% of the total urinary steroid fraction; hence estradiol-17β-17-sulfate derived from estradiol-17β-disulfate should be at best only a very minor component of the total urinary monosulfate fraction. In the present experiment estradiol-17β-3, 17-disulfate accounted for only about 2 percent of the total radioactive steroid fraction.

The systematic and (in brackets) trivial names of steroids mentioned in this paper are:-
3-hydroxy-estra-1, 3, 5(10)-trien-17-one (estrone):
estra-1, 3, 5(10)-triene-3, 17α-diol (estradiol-17α):
estra-1, 3, 5(10)-triene-3, 17β-diol (estradiol-17β):
estra-1, 3, 5(10)-triene-3, 16β, 17β-triol (16-epiestriol).

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SECTION F

METHODOLOGY FOR THE SEPARATION OF STEROID CONJUGATES

CHROMATOGRAPHIC SEPARATION OF ESTRONE AND 17β-ESTRADIOL CONJUGATES ON DEAE-SEPHADEX(1)

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ABSTRACT

17β-Estradiol-3-sulfate(E_2 3S), 17β-estradiol-17-sulfate (E_2 17S), 17β-estradiol-3-sulfate-17-glucosiduronate(E_2 3S17G) and 17β-estradiol-3,17-disulfate(E_2 3S17S), all labelled with ^3H, were prepared in the laboratory and identified by chromatographic, hydrolytic and crystallization techniques. Estrone-3-glucosiduronate(E_1 3G) and 17β-estradiol-3-glucosiduronate(E_2 3G), both labelled with ^{14}C, were also synthesized and similarly identified. Column chromatography on DEAE-Sephadex in a linear gradient of 0 - 0.8M NaCl, followed by 0.8 - 2.0M NaCl, resulted in a clear separation of 17β-estradiol(E_2), E_1 3G, 17β-estradiol-17-glucosiduronate (E_2 17G), estrone-3-sulfate(E_1 3S), E_2 3S, E_2 3S17G and E_2 3S17S. E_2 3S was not separable from E_2 17S under these conditions. The presence of urinary residues, while causing some changes in the elution pattern, still allowed of a reasonable separation. Some resolution of E_1 3G and E_2 3G was possible in a linear gradient (0 - 0.4M NaCl). Also, some separation could be obtained between E_2 3G and E_2 17G in a non-linear gradient (concave upwards; 0 - 1.0M NaCl).

INTRODUCTION

The use of DEAE-Sephadex as a means of separating certain groups of phenolic steroid conjugates has been described by Hahnel *etal* (4,5). We considered that this technique, in conjunction with other chromatographic procedures, might be particularly useful where a study is

made of the metabolism of a labelled compound such as 17β-estradiol-17-glucosiduronate($E_2$17G; 6), which in the human is known to give rise to little other than conjugates of estrone(E_1) and 17β-estradiol(E_2) in the urine (7). This lack of complexity in the pattern of metabolites might enable a relatively rapid identification of the major conjugates to be made if it were possible to extend the use of DEAE-Sephadex chromatography.

With the above in mind a number of glucosiduronate and sulfate conjugates of labelled E_1 and E_2 were prepared in our laboratory and, together with certain commercially available compounds, their behaviour on DEAE-Sephadex columns was investigated as described below.

MATERIALS

Amberlite XAD-2 resin was purchased from Rohm and Haas, Philadelphia, Pa., and was soaked in H_2O while sucking off suspended fines prior to use. DEAE-Sephadex (A-25; particle size 40 - 120μ) was purchased from Pharmacia (Canada) Ltd., Montreal, and was steeped in water for at least 24 hr, fine particles being removed by suction, prior to use. Celite was treated as described elsewhere (8).

Mylase P, containing phenolsulfatase, was obtained from Mann Research Labs., New York, and was used in incubations in 0.1M acetate buffer pH 6, overnight (9). Bacterial β-glucuronidase powder (Type II) was obtained from Sigma Chemical Co., St. Louis, Mo. It was used in 0.1M phosphate buffer, pH 7, at a concentration of 20 - 40 units/ml for a time of 24 hr (10).

Unlabelled steroids were purchased from Mann Research Labs. Estrone-6,7-^{3}H-3-sulfate($E_1$3S), specific activity (SA) = 7.8μc/μg and 17β-estradiol-6,7-^{3}H-17-glucosiduronate,

SA = 2.2μc/μg were purchased from New England Nuclear Corp., Boston, Mass., and were checked for purity as described earlier (7,10). 17β-Estradiol-6,7-^{3}H, SA = 147μc/μg, was also obtained from the latter source and was at least 98% pure as judged by crystallization with unlabelled carrier. Estrone-4-^{14}C, SA = 0.1μc/μg, was purchased from Radiochemical Centre, Amersham, Bucks, England, and was found to be radiochemically homogeneous on crystallization with carrier.

Adenosine-5'-triphosphate (ATP) was obtained as the disodium salt from Schwarz Bioresearch Inc., Orangeburg, N.Y. Uridine-5'-diphosphoglucuronic acid (UDPGA) was obtained as the ammonium salt from Sigma Chemical Co.

All solvents and reagents were of good quality and were further purified, where necessary, by published methods (8,11).

EXPERIMENTAL

Chromatographic methods

XAD-2 resin columns were employed to recover steroid conjugates from NaCl solutions following DEAE-Sephadex chromatography, and from urine to which they had been added. The salt solutions or urine were applied to the resin which was then washed with H_2O and eluted with methanol as described by Bradlow (12).

All Celite columns used were of dimensions 1 x 20 cm and each had 0.5 ml stationary phase per g Celite. The holdback volume (HBV) of these was ca. 9 ml. Steroid conjugates were transferred to the columns by dissolving in 0.5 - 0.75 ml methanol, mixing intimately with 0.5 g Celite, drying overnight in a vacuum desiccator and packing on top of the already prepared column. The solvent systems used were; system A - iso-octane:t-butanol:M NH_4OH (2:5:5), (13); system B - iso-octane:t-butanol:M NH_4OH (2:10:10),(14); system C - n-butanol:ethyl acetate:0.2% NH_4OH (3:1:4), (15).

DEAE-Sephadex, added as a slurry in H_2O, was allowed to settle under gravity in no. K9/60 Sephadex columns (Pharmacia Ltd.) to yield gel column dimensions of 0.9 x 50 cm. Pure steroid conjugates, and conjugates previously added to urine and recovered through XAD-2 resin, were applied in 5 ml H_2O

and washed into the column with a further 2 x 2.5 ml H_2O. Where whole pregnancy urine was to be chromatographed, 20 ml, diluted with 10 ml H_2O, were directly applied. Linear NaCl gradients were applied from two identical 1000 ml polyethylene bottles (Fisher Scientific Co., Montreal, Catalogue No. 2-962) connected by polyethylene tubing. The mixing vessel (complete with magnetic stirrer), delivering into the column, contained H_2O or the lesser of two NaCl concentrations. The linear systems used were; linear gradient 1 - 500 ml H_2O in mixing vessel, 500 ml 0.8M NaCl in reservoir vessel; linear gradient 2 - 500 ml 0.8M NaCl in mixing vessel, 500 ml 2.0M NaCl in reservoir; linear gradient 3 - 400 ml H_2O in mixing vessel, 400 ml 0.4M NaCl in reservoir. In addition, one non-linear gradient was used in which the mixing vessel contained 500 ml H_2O and the reservoir had 100 ml 1.0M NaCl (4). In this case the ratio of the surface area of the mixing vessel to that of the reservoir was 5:1.

Measurement of radioactivity

This was performed using either a dioxan scintillation fluid, or a toluene system in which the sample had already been dissolved in methanol. Details of this are published elsewhere (10).

Preparation of 17β-estradiol-6,7-^{3}H-3-sulfate($E_2$3S)

Purified ^{3}H-E_1 3S was reduced with $NaBH_4$ (16) and the product extracted with ether whereupon 1.2% of the initial ^{3}H was removed in the 'free' form. The conjugated material was extracted with n-butanol at pH 10 - 11 and partially purified by chromatography on Al_2O_3 in methanol containing a small concentration of NH_4OH (10). Further purification was achieved by DEAE-Sephadex chromatography in linear gradient 1 which yielded a single peak at ca. 0.45M NaCl, and by Celite chromatography (system **A**) in which a peak was eluted in HBV 3. In the latter system no more than 0.3% of the ^{3}H could be accounted for in an unconjugated form. The prepared material was hydrolyzed to an ether-soluble form by Mylase P to an extent of more than 95%. At least 98% of the ^{3}H was associated with a non-ketonic compound (Girard). Crystallization from methanol of 278,000 dpm (disintegrations /min.) with 26.8mg unlabelled E_2 yielded crystals (XLS) of **SA** = 10,800 dpm/mg and mother liquor (ML) of **SA** = 11,300 dpm/mg (calculated value = 10,400).

Preparation of 17β-estradiol-6,7-^{3}H-3,17-disulfate (E_2 3S17S)

^{3}H-E_2 was sulfurylated by the method of Fieser (17). After removal of free steroid with ether the conjugate was extracted into n-butanol (pH 10-11). It was then chromatographed on Al_2O_3 in methanol containing 0.15 (v/v)% NH_4OH yielding a single peak of radioactivity. This in turn was run on DEAE-Sephadex in linear gradient 1 followed by 2. About 1.5% of the applied ^{3}H was eluted in the position of monosulfate; the rest appeared as a symmetrical peak at ca. 1.4M NaCl. Celite chromatography in system A showed a single radioactive peak at about HBV 9. The disulfate was not extractable with ethyl acetate from unacidified 3M NaCl but became quantitatively extractable after Mylase P hydrolysis to yield ^{3}H-17β-estradiol-17-sulfate (E_2 17S). This latter, and the disulfate itself, were quantitatively solvolyzable in ethyl acetate-H_2SO_4 (18). The ^{3}H-E_2 released was crystallized with carrier from methanol (45,400 dpm + 18.1mg carrier; calc. SA = 2500 dpm/mg) to yield XLS and ML of SA = 2470 and 2480, respectively. When 48,000 dpm of the ^{3}H-E_2 3S17S were crystallized from methanol-ether with 28.1mg unlabelled carrier (synthesized in our laboratory) XLS and ML of SA = 1830 and 1890 dpm/mg, respectively, were obtained (calc. SA = 1710).

Preparation of 17β-estradiol-6,7-^{3}H-17-sulfate

^{3}H-E_2 17S was prepared by Mylase P hydrolysis of the labelled disulfate. It was purified by DEAE-Sephadex chromatography using linear gradient 1 and by Celite partition using system A. The purified compound was solvolyzed and the E_2 released identified by crystallization (see above).

Preparation of 17β-estradiol-6,7-^{3}H-3-sulfate-17-glucosiduronate (E_2 3S17G)

This was synthesized by incubating ^{3}H-E_2 17G for 1 hr at 37° C with a 105,000 x g supernatant fraction of female guinea pig liver in phosphate buffer (pH 6.5) to which had been added ATP, $MgSO_4$ and KCl (15,19). The incubation mixture was diluted with 4 vols. of ethanol, proteins were centrifuged off and washed, and the supernatant evaporated and chromatographed on DEAE-Sephadex in linear gradient 1. About 4% of the recovered ^{3}H was eluted at 0.14M NaCl, 9% at ca. 0.22M NaCl (100% hydrolyzable by β-glucuronidase;

presumably unchanged E_2 17G), and 87% at about 0.6M NaCl. The latter peak was applied to a Celite column which was developed with 14 HBV's of system A without elution of any radioactivity. Further elution with system B produced a rather wide peak between HBV's 6 and 13. Rechromatography on Celite in system C resulted in elution of the ^3H between HBV's 3 and 5. An aliquot of the latter material was incubated with Mylase P resulting in <1% of the ^3H being recoverable in an ether-soluble form. The water-soluble material from this incubation was eluted at HBV 7 from Celite in system A (i.e. typical of a monoglucosiduronate). This, in turn was cleaved (>98%) by β-glucuronidase. A further aliquot of the presumptive mixed conjugate was incubated with β-glucuronidase (<1% ^3H converted to ether-soluble form) and the H_2O-soluble product shown to be eluted from Celite in system A in HBV 3 (i.e. typical of a monosulfate). Incubation with Mylase P yielded 97% hydrolysis. The ether-soluble radioactivity from the two sets of incubations were pooled and an aliquot (406,000 dpm) was crystallized from methanol with 23.8mg unlabelled E_2 (calc. SA = 17,000 dpm/mg). XLS and ML, each of SA = 16,800, were obtained. The XLS were acetylated and crystallized from methanol-H_2O to yield SA values of 12,900 and 13,200 dpm/mg for XLS and ML, respectively (calc. SA = 12,800).

Preparation of estrone-4-^{14}C-3-glucosiduronate (E_1 3G)

^{14}C-E_1 was incubated with a whole homogenate of female rabbit liver in phosphate buffer, pH 7.5, with added UDPGA, under the conditions described by Levitz *et al* (20) for the biosynthesis of estriol-3-glucosiduronate. A deproteinized extract of the incubation mixture was chromatographed in linear gradient 1 on DEAE-Sephadex whereupon 83% of the recovered ^{14}C was eluted at about 0.15M NaCl and 17% slightly later. The latter was completely hydrolyzed by β-glucuronidase and the steroid released shown to be 100% non-ketonic. The major peak was chromatographed on Celite in system A and eluted in HBV 7 - 10. It also was quantitatively hydrolyzed by β-glucuronidase and at this stage the ^{14}C-steroid released was 85 - 90% ketonic. Further DEAE-Sephadex chromatography was performed using the non-linear gradient (0 - 1.0M NaCl). A sharp peak of radioactivity was eluted at about 0.15M NaCl. Following β-glucuronidase hydrolysis an aliquot of the free material (65,000 dpm) was crystallized thrice from methanol with 22.3mg unlabelled E_1 (calc. SA = 2920 dpm/mg). The

third XLS and ML had SA values of 2730 and 2700, respectively. These XLS were acetylated and crystallized twice from methanol-H_2O. The second XLS and ML had SA = 2290 and 2260 dpm/mg, respectively (calc. SA = 2360).

Preparation of 17β-estradiol-4-^{14}C-3-glucosiduronate (E_2 3G)

This was prepared by the reduction of ^{14}C-E_1 3G with $NaBH_4$ in methanol. The conjugate was recovered by treatment with XAD-2 resin and was then subjected to DEAE-Sephadex chromatography in linear gradient 3 and then in the non-linear gradient. In each case sharp peaks of radioactivity were noted. Hydrolysis with β-glucuronidase was quantitative and an aliquot of the released ^{14}C (54,500 dpm) was crystallized thrice from methanol with 26.7mg unlabelled E_2 (calc. SA = 2040 dpm/mg). The third XLS and ML had SA values of 1930 and 1960, respectively. The last XLS were acetylated and crystallized twice from methanol-H_2O. The second XLS and ML obtained had SA = 1460 and 1450 dpm/mg, respectively (calc. value = 1490).

RESULTS

The 3-monosulfates of E_1 and E_2 were well separated by DEAE-Sephadex chromatography using a gradient of 0 - 0.8M NaCl (Fig. 1). This was so whether these two compounds were chromatographed in the pure state, added to 20 ml pregnancy urine diluted with 10 ml H_2O and applied directly to the column, or added to one-third of a 24 hr non-pregnancy urine, and eluted from the XAD-2 resin prior to chromatography. Widening of the peaks did occur in the presence of urine residues. Thus, in the pure state E_1 3S was recovered in 50 - 60 ml eluate and E_2 3S in 60 - 80 ml. Following addition to urine these values were 80 - 100 ml and 90 - 120 ml, respectively. The elution pattern of the two

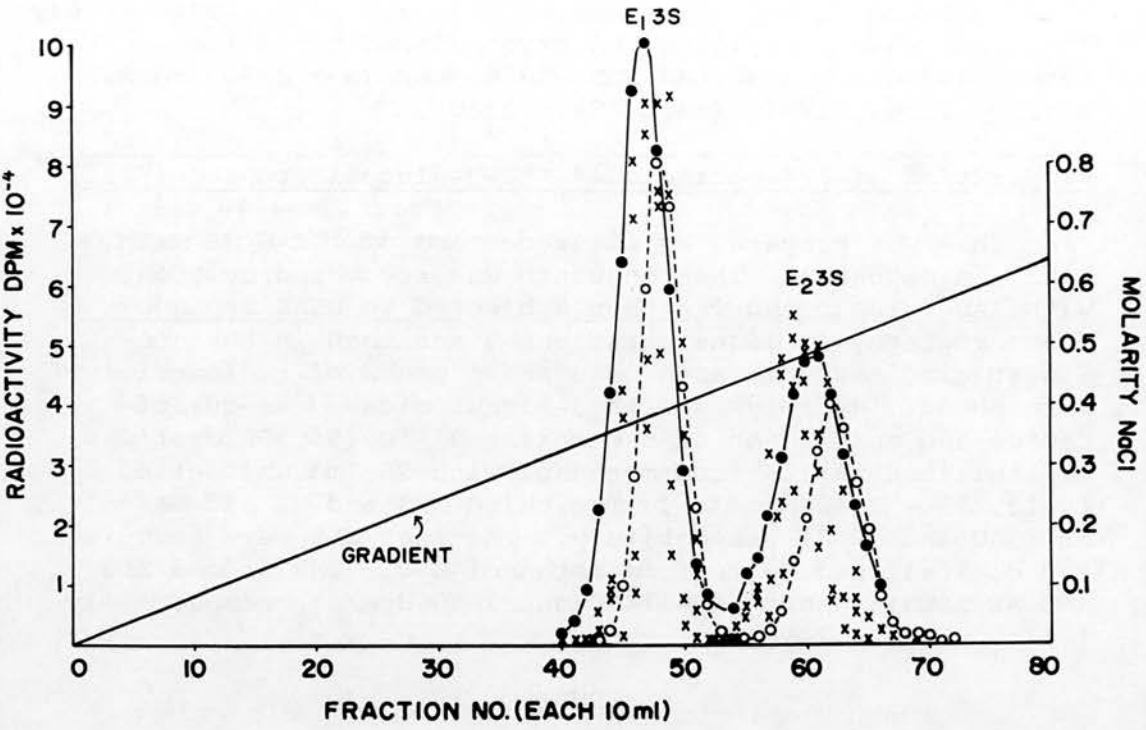

Fig. 1. DEAE-Sephadex chromatography (0 - 0.8M NaCl, linear gradient) of ^{3}H-E_1 3S and ^{3}H-E_2 3S: x - elution pattern of pure conjugates in 4 separate experiments; o - elution pattern of conjugates added to 20 ml late preg. urine diluted with 10 ml H_2O; ● - elution pattern of conjugates added to 1/3 of a 24 hr non-preg. urine and processed on Amberlite XAD-2.

sulfates was quite consistent from one experiment to another (Table I) and recovery from the Sephadex columns was always in excess of 95%. When the separated peaks were hydrolyzed with Mylase P it was shown that E_1 was the sole constituent of the first eluted and E_2 of the second peak, thus establishing the relative positions of E_1 3S and E_2 3S.

TABLE I

Elution of E_1 3S and E_2 3S from DEAE-Sephadex by a linear gradient of 0 - 0.8M NaCl

Experiment	Fractions (10 ml) containing conjugates	
	E_1 3S	E_2 3S
E_1 3S + E_2 3S (pure)	44 - 49	55 - 62
" " "	46 - 51	57 - 64
" " "	46 - 50	57 - 63
" " "	45 - 49	57 - 62
E_1 3S + E_2 3S + 20 ml late preg. urine diluted with 10 ml H_2O	45 - 52	58 - 66
E_1 3S + E_2 3S + 1/3 of a 24 hr non-preg. urine processed on XAD-2	42 - 51	55 - 66

The linear gradient 0 - 0.8M NaCl also had a wide applicability in the separation of several other E_1 and E_2 conjugates. Fig. 2 shows the elution pattern of a mixture of purified ^{14}C-E_2, ^{14}C-E_1 3G, ^{3}H-E_2 17G, ^{3}H-E_1 3S, ^{3}H-E_2 3S and ^{3}H-E_2 3S17G. These six compounds were clearly separable from each other. Superimposed upon this is the pattern for the five above-mentioned conjugates plus ^{14}C-E_2 3G, all of which had been added to one-third of a 24 hr non-pregnancy urine and processed via the XAD-2 resin. The gradient employed was not capable of separating E_1 3G and E_2 3G, the latter appearing as a shoulder on the peak of the former. Certain shifts in the elution pattern were presumably caused by the presence of urine residues; the most marked occurring

in the case of $E_2$3S17G which was eluted earlier than in the pure state, resulting in a poorer separation between it and $E_2$3S. $E_2$3S17S was not eluted over the range 0 - 0.8M NaCl but appeared as a symmetrical peak between concentrations of ca. 1.2 and 1.6M NaCl in a more concentrated system. In all instances recovery of the added conjugates from the DEAE-Sephadex column was at least 95%.

Table II shows the NaCl concentrations at which the various compounds were eluted. $E_2$17S (not shown in the Figs.) was apparently not separable from $E_2$3S in the 0 - 0.8M NaCl system.

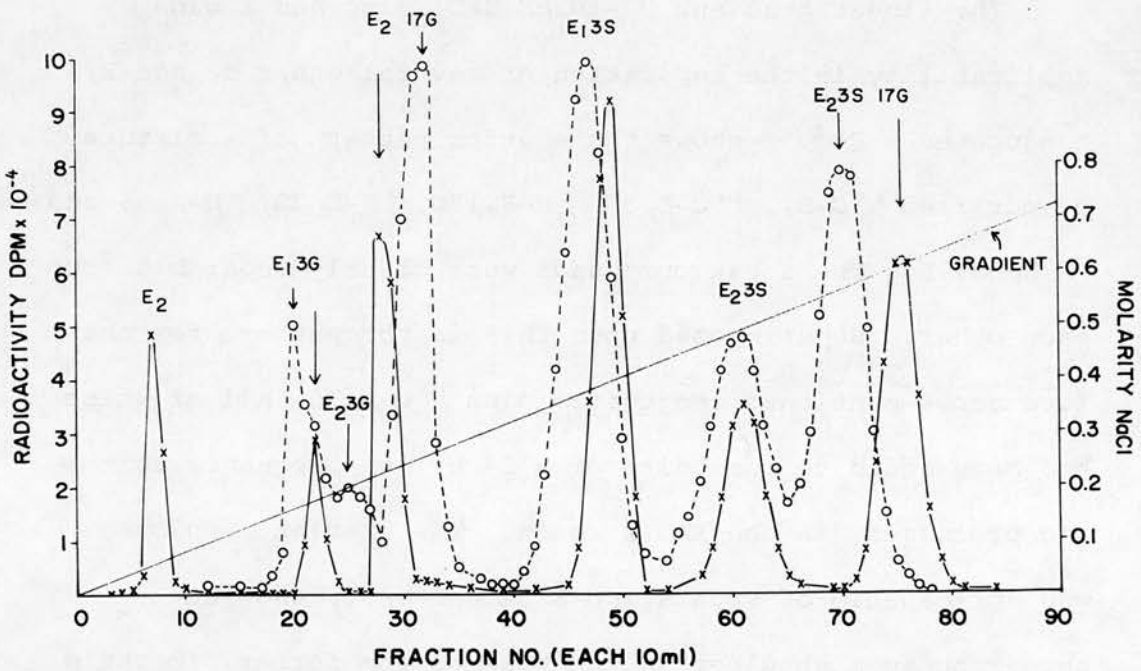

Fig. 2. DEAE-Sephadex chromatography of compounds (0 - 0.8M NaCl linear gradient): x - pure ^{14}C-E_2, ^{14}C-$E_1$3G, ^{3}H-$E_2$17G, ^{3}H-$E_1$3S, ^{3}H-$E_2$3S and ^{3}H-$E_2$3S17G; o - ^{14}C-$E_1$3G, ^{14}C-$E_2$3G, ^{3}H-$E_2$17G, ^{3}H-$E_1$3S, ^{3}H-$E_2$3S and ^{3}H-$E_2$3S17G added to 1/3 of a 24 hr non-preg. urine and processed on Amberlite XAD-2.

TABLE II

Salt concentrations required for elution of pure compounds from DEAE-Sephadex using a linear gradient of 0 - 0.8M NaCl followed by 0.8 - 2.0M NaCl

Compound	NaCl concentration range (M)
E_2	.05 - .07
E_1 3G	.17 - .19
E_2 3G	.18 - .23 (approx.)
E_2 17G	.22 - .25
E_1 3S	.37 - .41
E_2 3S	.46 - .52
E_2 17S	.46 - .52
E_2 3S17G	.57 - .64
E_2 3S17S	1.00 -1.20

Variation of the NaCl gradient achieved some, though not complete, separation of E_1 3G from E_2 3G and of E_2 3G from E_2 17G. Fig. 3 shows the pattern obtained for the former two conjugates in the linear gradient 0 - 0.4M NaCl, and Fig. 4 shows the separation achieved of the two E_2 conjugates in the non-linear gradient (0 - 1.0M NaCl).

DISCUSSION

The labelled conjugates synthesized in our laboratory for use in this study appeared, on the whole, to possess the required degree of radiochemical homogeneity.

It is clear from the results presented above that DEAE-Sephadex possesses a great potential for the separation of phenolic steroid conjugates. Thus, not only does there exist the possibility of separating like steroids conjugated

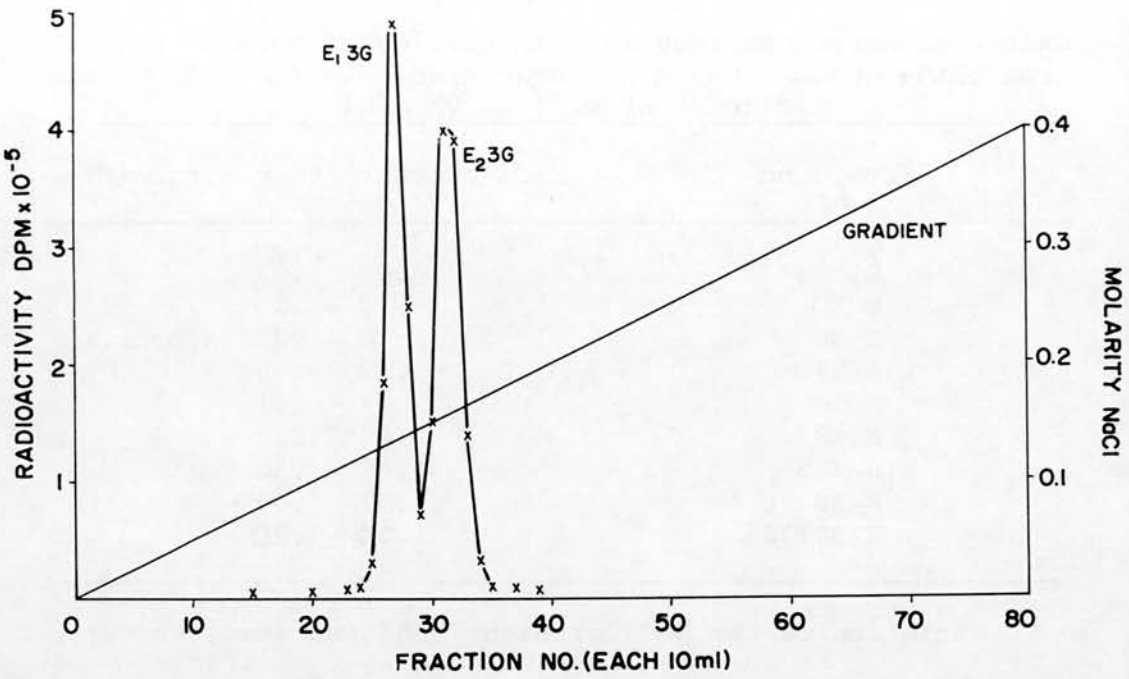

Fig. 3. DEAE-Sephadex chromatography of pure ^{14}C-E_1 3G and ^{14}C-E_2 3G in 0 - 0.4M NaCl, linear gradient.

with the same acid at different carbon atoms of the steroid molecule (4,5), but also the very clear resolution of steroids differing only slightly in structure and conjugated with the same acid at the same carbon atom. This is particularly evident in the simple separation of E_1 3S and E_2 3S and to a lesser extent for the 3-glucosiduronates of E_1 and E_2. The excellent separation of the two monosulfates suggests that more than simple ion-exchange of the sulfate group is involved. There can be no doubt about the ease

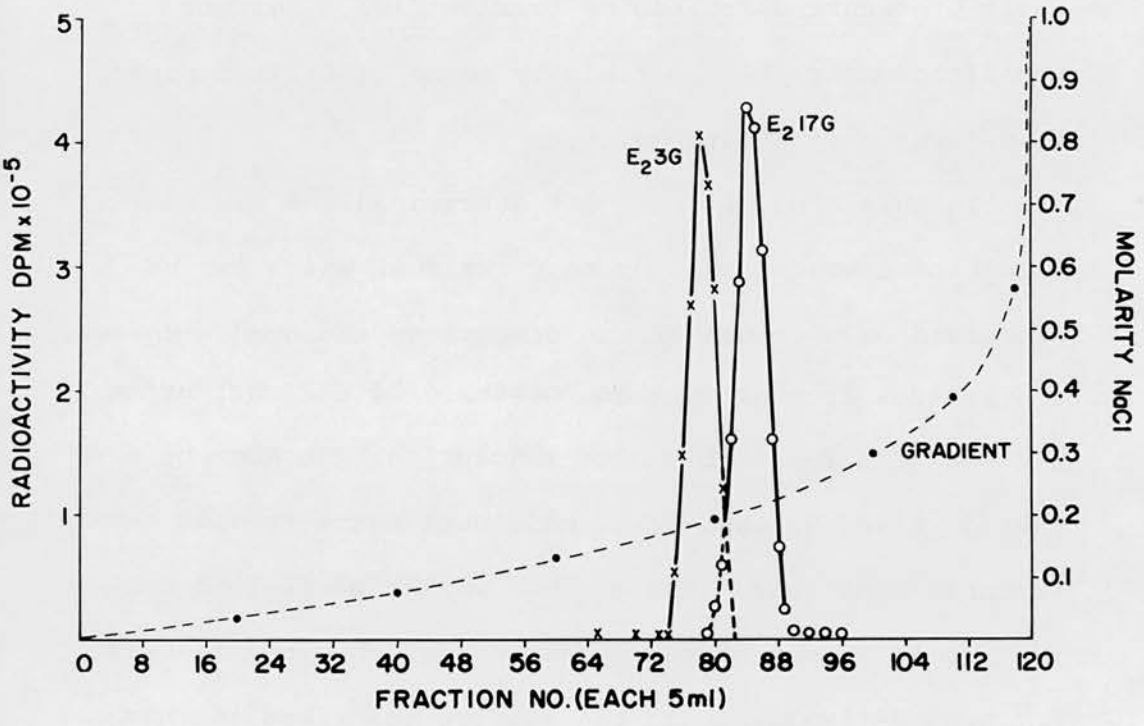

Fig. 4. DEAE-Sephadex chromatography of pure ^{14}C-E_2 3G and ^{3}H-E_2 17G in 0 - 1.0M NaCl, non-linear gradient.

with which monoglucosiduronates, monosulfates, mixed conjugates and double conjugates may be separated on a linear gradient of NaCl. This finding apparently differs in some important respects from the findings of Hahnel et al who experienced problems in separating monosulfates from 16-, or 17-monoglucosiduronates (4,5).

Elution of the conjugates in aqueous salt solution does not represent a major inconvenience since they may be recovered, more or less salt-free, by the elegant XAD-2

resin procedure described by Bradlow (12). Further purification is then possible by means of Celite, paper, thin-layer chromatography, etc.

In this study we have not determined the maximum amount of extract (e.g. urinary residue) which can be processed on a column of the dimensions employed. However, the residue from as much as one-third of a 24 hr. urine allowed of a remarkably good resolution. It must be expected, nevertheless, that individual extracts will exhibit variable behaviour. The maximum weight of steroid conjugates which may be safely chromatographed has likewise not been determined. In the studies described in this paper very small amounts were involved except where pregnancy urine was used in which case some 400μg of 'total estrogens' were chromatographed without marked effect (at least on the pattern of E_1 3S and E_2 3S).

While it is clear that many conjugates of other phenolic steroids are naturally-occurring and that these would, if present, interfere to varying extents with the compounds studied in the present work (4,5), chromatography on DEAE-Sephadex offers a useful means of studying the metabolism and interconversion of E_1 and E_2 conjugates where no important contribution is made by other phenolic steroids. In this respect the <u>in vitro</u> renal metabolism

of E_1 and E_2 conjugates has recently been investigated, using this technique, in our laboratory (21).

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Separation of Monoglucosiduronate Conjugates of Estrone and 17β-Estradiol by DEAE-Sephadex Chromatography[1]

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In an earlier publication from this laboratory (1) the separation of monoglucosiduronate, monosulfate, sulfoglucosiduronate, and disulfate conjugates of estrone (E_1)[3] and/or 17β-estradiol (E_2) was described. The technique involved elution of DEAE-Sephadex (A 25) columns with simple concentration gradients of NaCl in H_2O and was based on the original studies of Hahnel (2). It was also pointed out (1) that a certain degree of separation of estrone-3-glucosiduronate ($E_1$3G) and 17β-estradiol-3-glucosiduronate ($E_2$3G) was possible, while the latter compound could be partially separated, in turn, from 17β-estradiol-17-glucosiduronate ($E_2$17G). More recently, continued experience with the above method has resulted in practically complete separation of those three monoglucosiduronates from each other. This procedure has proved to be of particular value in the study of estrogen glucosiduronate metabolism in the human (3, 4). The present report concerns the exact methodology now being applied in our laboratory and the results which may be expected from it.

MATERIALS

17β-Estradiol-6,7-^{3}H-17-glucosiduronate, specific activity (SA) = ca. 1 mCi/μmole, was purchased from New England Nuclear Corp., Boston, Mass., and was purified as described elsewhere (5). The 3-gluco-

[1] Supported by grant MT 532 from The Medical Research Council of Canada.

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[3] The following trivial names and abbreviations are used: estrone (E_1) = 3-hydroxyestra-1,3,5(10)-trien-17-one; 17β-estradiol (E_2) = estra-1,3,5(10)-triene-3,17β-diol; estrone-3-glucosiduronate ($E_1$3G) = 17-oxoestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-3-glucosiduronate ($E_2$3G) = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-17-glucosiduronate ($E_2$17G) = 3-hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate.

siduronates of estrone-6,7-^{3}H and 17β-estradiol-6,7-^{3}H were synthesized and purified as described earlier (1). Estrone-4-^{14}C, SA = ca. 27 μCi/μmole, and 17β-estradiol-6,7-^{3}H, SA = ca. 48 mCi/μmole, were both purchased from New England Nuclear Corp.

Sephadex columns (K 9/60) of internal diameter 0.9 cm were purchased from Pharmacia (Canada) Ltd., Montreal, as was Sephadex A 25 of particle size 40–120 microns.

Liquid scintillation fluid, consisting of a toluene solution of 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (butyl-PBD), was purchased as a concentrate from Nuclear-Chicago Corp. (a subsidiary of G. D. Searle & Co.) and diluted, prior to use, with toluene, to give a concentration of 7 gm/liter.

All other chromatographic materials, chemicals, and organic solvents were obtained and purified where necessary by published techniques (6).

METHODS

Column preparation. Sephadex A 25 was mixed with distilled H_2O [4] in the approximate proportion of 10 gm/150 ml. This mixture was stirred at least six times, at intervals, and allowed to settle each time for about 30 min prior to sucking off the supernatant and suspended fines and replacing with H_2O prior to stirring again. The gel so prepared was normally used to pack columns the following day, or later. The tip of the column was fitted with a short piece of rubber tubing which was closed with a clip and the column filled to within 4 or 5 cm of the top with H_2O. The A 25 suspension was thoroughly stirred and an aliquot removed by Pasteur pipet fitted with a rubber teat. The tip of the pipet was introduced under the H_2O surface in the column and the slurry expelled. In this way the column was filled. When the upper 4–5 cm of the column H_2O had cleared of A 25 it was removed by Pasteur pipet and replaced by a similar volume of the slurry. This was continued, with frequent manual rotation of the column, until one-third to one-half of the column length was filled with packed A 25 gel. The clip was then removed and the column allowed to flow freely while continuing the addition of the slurry, as above, until a bed height of 58 cm was obtained. (Several columns can, of course, be prepared simultaneously.) On occasion the prepared column was attached to a reservoir of H_2O and allowed to run overnight, although immediate use for chromatography of steroid conjugates was found to be entirely successful. Columns were not reused in this work.

The total bed volume of such a column was 37 ml and each gram of

[4] No difference was noted between H_2O which had been distilled once before use and that distilled once and then passed through a mixed-bed ion-exchange resin.

original dry A 25 occupied a packed volume of ca. 8 ml. Under a head of 50 cm of H_2O the flow rate varied between 40 and 50 ml/hr from one column to another.

Preparation of samples for chromatography. Pure labeled steroids or their conjugates were dissolved in 3–5 ml of H_2O with brief warming in a water bath at 50°C. Urine aliquots were chromatographed on Amberlite XAD-2 resin[5] and the conjugated steroid fraction recovered as described by Bradlow (7). The conjugate fraction was dissolved in H_2O as for the pure compounds, and glucosiduronates of labeled E_1 and E_2 were added prior to A 25 chromatography. Where compounds were chromatographed on one column and were to be rerun on another, they were recovered from aqueous solution by Amberlite XAD-2 chromatography, as above. For purposes of further purification, urinary fractions, initially chromatographed on one A 25 column, were run on 1×20 cm Celite partition columns in the system isooctane/*tert*-butanol/1 *M* NH_4OH (2:5:5) (1, 8) prior to rechromatography on a second Sephadex column.

A 25 chromatography. This was performed at a room temperature of 22–24°C although no attempt was made to create a thermostatically controlled environment. Aqueous solutions (3–5 ml) were applied carefully, in portions, by pipet, until all had been added. During this time the column was open but special care was taken to ensure that the top of the packed gel never became dry. The vessel which had contained the fraction to be chromatographed was then successively washed with 2×2 or 3 ml of H_2O and this was also added to the column. The column was then attached to a gradient system of two identical 1000 ml bottles (Fisher Scientific Co., Montreal, Canada, catalog #2-962—see ref. 1) connected by a short piece of polyethylene tubing, and one of these, the mixing vessel, was stirred magnetically. From this latter vessel a tube carried the eluting fluid to the column. Three gradient systems were employed: (*a*) 400 ml of H_2O in the mixing vessel, 400 ml of 0.2 *M* NaCl in the donor vessel (gradient 0–0.2 *M* NaCl); (*b*) 400 ml of H_2O in the mixing vessel, 400 ml of 0.4 *M* NaCl in the donor vessel (0–0.4 *M* NaCl); (*c*) 500 ml of H_2O in the mixing vessel, 500 ml of 0.8 *M* NaCl in the donor vessel (0–0.8 *M* NaCl). The column was allowed to flow freely under a head of ca. 50 cm of eluting solution, the rubber tip having been removed. Suitable fractions (3–10 ml) were collected. At the completion of the 0–0.4 *M* NaCl gradient the gel bed showed a 24% shrinkage. A 31% shrinkage occurred in the 0–0.8 *M* NaCl gradient.

Analysis of eluted fractions. Radioactivity was assayed on small aliquots (suitably dried) of fractions by counting in 0.5 ml of methanol

[5] Rohm and Haas, Philadelphia, Pa.

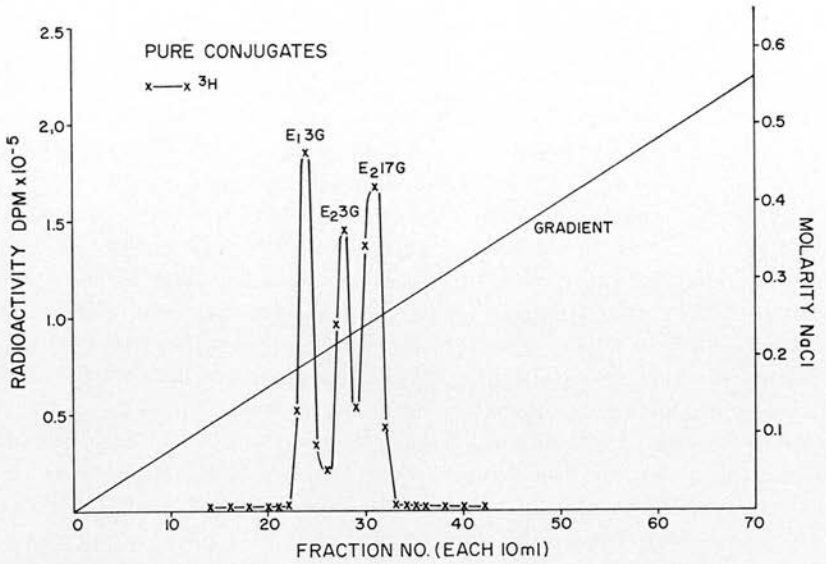

FIG. 1. Chromatography of pure ³H-labeled $E_1$3G, $E_2$3G, and $E_2$17G on A 25 in a linear gradient of 0–0.8 *M* NaCl.

and 10 ml of butyl-PBD in toluene, in a Nuclear-Chicago liquid scintillation spectrometer as described elsewhere (9). Quenching was corrected for by the internal standard technique. Optical densities of urinary chromogens were measured in a Unicam SP 600 spectrophotometer.

RESULTS

Figures 1 and 2 show the elution patterns obtained by successively chromatographing a mixture of pure ³H-labeled $E_1$3G, $E_2$3G, and $E_2$17G on A 25 in 0–0.8 *M* NaCl and then on a second column in 0–0.4 *M* NaCl. A partial separation of the compounds was seen in the first gradient (Fig. 1) while in the second gradient practically complete separation was achieved (Fig. 2).

When the same three compounds were added to a 6 hr aliquot of human female (nonpregnant) urine the pattern obtained in the 0–0.8 *M* NaCl gradient is that shown in Figure 3. Much of the urinary chromogen was eluted prior to the monoglucosiduronate fractions, resulting in considerable purification. A considerable fraction of chromogen was firmly adsorbed at the top of the A 25 column. During Celite partition chromatography of the pooled glucosiduronate fractions of Figure 3, little or no chromogen was eluted with the ³H-label (9–10 holdback volumes). This purified material, chromatographed on A 25 in 0–0.4 *M* NaCl yielded the pattern shown in Figure 4.

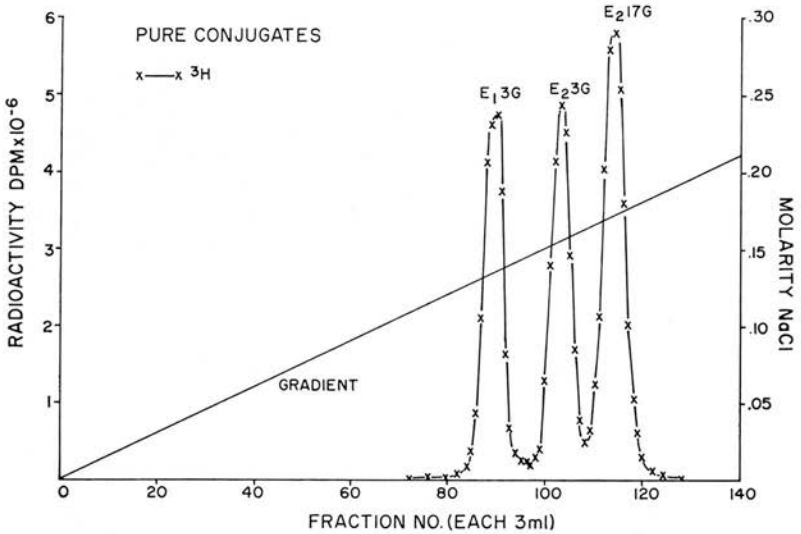

FIG. 2. Chromatography of pure ^{3}H-labeled $E_1$3G, $E_2$3G, and $E_2$17G on A 25 in a linear gradient of 0–0.4 *M* NaCl. Compounds had already been run in 0–0.8 *M* NaCl.

Table 1 contains data for the NaCl concentrations[6] (read directly from the linear gradient curves) at which the conjugates were eluted. It did not appear that the presence of urine residues from up to 9 hr urine volumes untowardly affected the behavior of the three compounds in

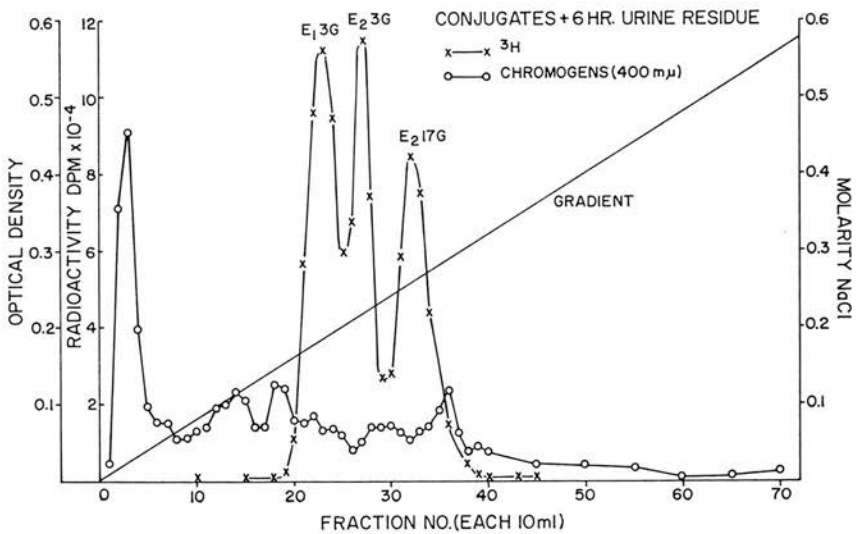

FIG. 3. Chromatography of ^{3}H-labeled $E_1$3G, $E_2$3G, and $E_2$17G, initially added to a 6 hr urine extract, on A 25 in 0–0.8 *M* NaCl.

[6] Salt concentrations were calculated on theoretical grounds.

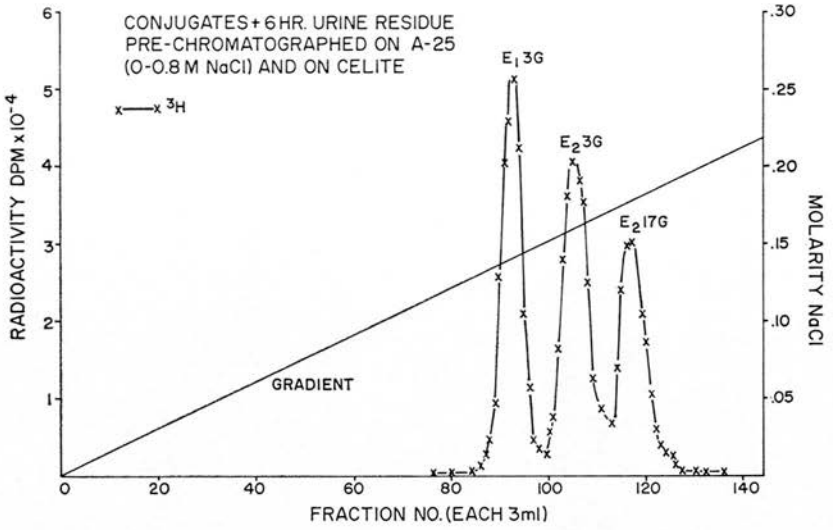

FIG. 4. Chromatography of ^{3}H-labeled $E_1$3G, $E_2$3G, and $E_2$17G, initially added to a 6 hr urine extract, on A 25 in 0–0.4 *M* NaCl, after prechromatography in 0–0.8 *M* NaCl and on Celite.

question. The average eluate volume between the $E_1$3G and $E_2$3G peaks was 40 ml in either gradient and the corresponding value for $E_2$3G and $E_2$17G was 35 ml. The NaCl concentration required to elute any given monoglucosiduronate was higher for the 0–0.8 *M* NaCl gradient than for the 0–0.4 *M* NaCl system. This may relate to a combination of factors

TABLE 1
NaCl Concentrations for Elution of Estrogen Glucosiduronates from A 25 Columns

Chromatography conditions	Molarity of NaCl for elution of peak fractions			No. of expts.
	$E_1$3G	$E_2$3G	$E_2$17G	
Pure conjugates (0–0.8 *M* NaCl)	0.190	0.223	0.247	1
Conjugates in urine[a] (0–0.8 *M* NaCl)	0.193 (.187–.200)	0.224 (.216–.240)	0.257[b]	3
Pure conjugates (0–0.4 *M* NaCl)	0.139 (.135–.144)	0.160 (.155–.164)	0.177 (.172–.184)	3
Conjugates in urine[c] (0–0.4 *M* NaCl)	0.144 (S.D. = .010)	0.164 (S.D. = .011)	0.176[b]	10

[a] Prechromatographed on Amberlite XAD-2 resin.

[b] $E_2$17G run in one experiment.

[c] Prechromatographed on Amberlite XAD-2 resin, A 25 (0–0.8 *M* NaCl), and Celite; conjugates added to 3–9 hr urine volumes.

such as the rate of change of salt concentration and the relatively high flow rate of the columns.

Table 2 shows data for the behavior of ^{14}C-E_1 and ^{3}H-E_2 on A 25 in four different systems. These two compounds were almost completely separated as sharp peaks occurring at about 70 and 85 ml, respectively, of column eluate in all of the systems. Thus the NaCl concentration played no obvious role in the elution or separation of these compounds.

TABLE 2
Elution of Unconjugated E_1 and E_2 from A 25 Columns[a]

Eluting system	^{14}C–E_1		^{3}H–E_2	
	Eluate vol. at peak (ml)	NaCl at peak (*M*)	Eluate vol. at peak (ml)	NaCl at peak (*M*)
H_2O	65	—	80	—
0–0.2 *M* NaCl	70	0.018	85	0.021
0–0.4 *M* NaCl	70	0.035	85	0.043
0–0.8 *M* NaCl	70	0.070	85	0.086

[a] 5 ml fractions collected in each case.

DISCUSSION

It is clear that linear concentration gradients of NaCl can separate, more or less completely, the 3-glucosiduronates of E_1 and E_2 and the 17-glucosiduronate of E_2. The separation of 3- from 16- or 17-glucosiduronates of phenolic steroids using nonlinear gradients was reported by Hahnel (10). The separation achieved in the present study was reasonably reproducible and has been of particular value in the study of the metabolism of labeled $E_2$17G (3), and $E_1$3G and $E_2$3G (4), in the human. In our hands urinary E_1 and E_2 monoglucosiduronates can be highly purified by the above techniques when performed exactly as described. The use of the 0–0.8 *M* NaCl gradient allows of great purification, rather than separation, while the 0–0.4 *M* NaCl gradient results in separation. However, it should be pointed out that omission of the Celite chromatographic step between the two A 25 columns can lead to unsuccessful separations in the 0–0.4 *M* NaCl system. Also, although urine residues up to 9 hr of nonpregnancy urine have been successfully analyzed, the over-all performance benefits from the use of 6 hr, or less, of original urine.

It was suggested elsewhere (1) that the separation of $E_1$3G and $E_2$3G was unlikely to depend solely on ion exchange of the carboxyl group of the glucosiduronate moiety. This is supported by the separation of E_1 and

E_2 in a manner independent of NaCl concentration. This would indicate that certain groupings in ring D allow of separation through some "adsorptive" process, not clearly defined. The later elution of the glucosiduronates than of the free steroids presumably relates to the presence of the ionized carboxyl groups, and the greater degree of separation between $E_1$3G and $E_2$3G, than between E_1 and E_2, to the slower elution of the glucosiduronates. Thus the behavior of $E_1$3G and $E_2$3G on A 25 depends upon the presence of the glucosiduronate moiety, differences in ring D, and probably other factors (unpublished observations made in this laboratory show that neutral steroid conjugates are eluted more quickly than phenolic steroid conjugates). It should be noted that the 3-sulfates of E_1 and E_2, which are eluted much later than $E_1$3G and $E_2$3G, are in turn separated to a greater degree than are the glucosiduronates (1).

SUMMARY

The application of NaCl gradient elution of DEAE-Sephadex columns (0.9×58 cm) is described in detail for the separation of ^{3}H-labeled estrone-3-glucosiduronate, 17β-estradiol-3-glucosiduronate and 17β-estradiol-17-glucosiduronate. When these three compounds were added to urine, considerable purification was achieved in a linear gradient of 0–0.8 *M* NaCl. Chromatography of the monoglucosiduronate fractions on Celite columns, followed by a linear gradient (0–0.4 *M* NaCl) on a further DEAE-Sephadex column, resulted in good separation of the three conjugates from each other as colorless fractions.

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Behaviour of dehydroisoandrosterone, testosterone and their conjugates on DEAE-Sephadex

Recent publications have shown the value of DEAE-Sephadex chromatography in separating several types of phenolic steroid conjugates[1–4]. The present report concerns the behaviour of two neutral C_{19} steroids and their conjugates in simple NaCl concentration gradients on DEAE-Sephadex columns.

Experimental

Reagents. DEAE-Sephadex (A-25) of medium porosity was purchased from Pharmacia (Canada) Ltd., Montreal, Quebec, and packed to yield columns of 58 × 1 cm as described elsewhere[4].

Steroids and conjugates. [7-^{3}H]Dehydroisoandrosterone (DHA) of specific activity (S.A.) 1.6 Ci/mmole was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. and [4-^{14}C]testosterone (T) of S.A. 29.2 Ci/mole was purchased from Radiochemical Centre, Amersham, Bucks., Great Britain (now Amersham-Searle). The conjugates were purchased from New England Nuclear Corp. These were [7-^{3}H]dehydroisoandrosteron-3-yl-β-D-glucopyranosiduronate (DHAG) of S.A. 10 Ci/mmole, the NH_4^+ salt of [7-^{3}H]dehydroisoandrosteron-3-yl-sulphate (DHAS) of S.A. 10 Ci/mmole, [1,2-^{3}H]testosteron-17-yl-β-D-glucopyranosiduronate (TG) of S.A. 50 Ci/mmole, and the NH_4^+ salt of [7-^{3}H]testosteron-17-yl-sulphate (TS) of S.A. 8 Ci/mmole.

Methods. Aqueous solutions of various mixtures of the above labelled compounds, containing a drop of methanol to facilitate solution of the free steroids, were applied to DEAE-Sephadex columns which were developed with linear concentration gradients of NaCl in H_2O as described elsewhere[2,4]. The mixing vessel in each case contained 400 ml H_2O and the donor vessel contained 400 ml of either 0.2, 0.3, 0.4 or 0.8 *M* NaCl. 5-ml fractions of eluate were collected and radioactivity was determined by liquid scintillation spectrometry[5].

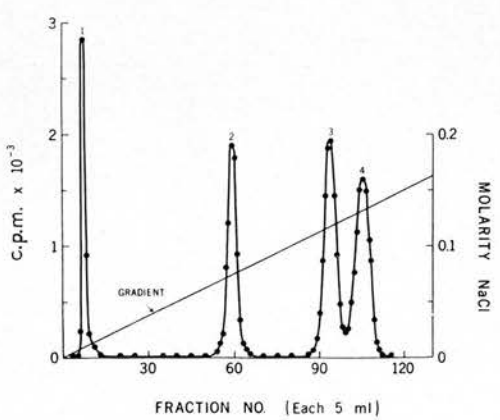

Fig. 1. DEAE-Sephadex chromatography in a linear gradient (0–0.2 *M* NaCl) of: peak 1, DHA or T; peak 2, DHAG or TG; peak 3, TS; peak 4, DHAS.

Results and discussion

In none of the gradients could DHA be separated from T nor could DHAG be separated from TG. However, the free steroids were easily separable from the glucosiduronates in all gradients. The sulphates, in turn, were well separated from the free steroids and glucosiduronates and, furthermore, DHAS and TS were separable from each other to varying extents in the different gradients. The greatest degree of separation between the two sulphates occurred in the 0–0.2 *M* NaCl gradient (Fig. 1) and in the 0–0.3 *M* gradient. The chromatographic mobilities of DHA and T were independent of the gradient employed but, as expected, behaviour of the conjugates was dependent on salt concentration. The sulphates of DHA and T were eluted in positions similar to certain oestrogen monoglucosiduronates[2,4] and considerably before oestrogen monosulphates[2].

It is of interest to note that when, in one experiment, employing the 0–0.2 *M* NaCl gradient, labelled oestrone and 17β-oestradiol were chromatographed along with ^{3}H-DHA, the oestrogens were eluted between fractions 12 and 20 while DHA appeared between fractions 6 and 11. It would appear from this and from the behaviour of the conjugates that the presence of a phenolic group allows a greater retention by the DEAE-Sephadex (see ref. 4).

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SECTION G

METABOLISM OF OESTRONE AND OESTRADIOL GLUCOSIDURONATES IN THE HUMAN IN VIVO

METABOLISM OF 17β-ESTRADIOL-6,7-^{3}H-17-GLUCOSIDURONATE BY THE HUMAN FEMALE(1)

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ABSTRACT

17β-Estradiol-6,7-^{3}H-17-glucosiduronate was injected intravenously into 2 normal young females. In one, 17β-estradiol-4-^{14}C was simultaneously administered by the same route. Most of the ^{3}H was excreted in 48 hr., the main urinary metabolites being estrone and 17β-estradiol which were liberated from conjugation by β-glucuronidase hydrolysis. These 2 steroids occurred in a ratio of about 1:1 and accounted for more than 80% of identified ^{3}H-metabolites. Estriol accounted for only 3% and other 16-oxygenated metabolites were of minor quantitative significance as was 2-methoxyestrone. Smaller amounts of each steroid were liberated from conjugation by solvolysis after β-glucuronidase incubation. When 17β-estradiol-4-^{14}C was administered along with the glucosiduronate, urinary 2-methoxyestrone, estrone and 17β-estradiol 'glucosiduronates' were produced to a greater extent from the injected conjugate than from the free steroid. This was also true of 17β-estradiol liberated by solvolysis. 2-Methoxyestrone and estrone liberated by solvolysis had ^{3}H/^{14}C ratios similar to that injected whereas all of the 16-oxygenated metabolites, regardless of conjugation, were formed preferentially from injected 17β-estradiol.

INTRODUCTION

Considerable interest has been evident over the last few years in the metabolism of steroid conjugates. It is generally considered that steroid glucosiduronates are formed mainly, if not wholly, for purposes of excretion in bile and urine. Sandberg and Slaunwhite have claimed that injected estriol-16-glucosiduronate(3) is not metabolized by the human but rather quantitatively excreted in the urine(4). Recent work by Diczfalusy et al has indicated the formation of estriol-3-glucosiduronate from injected estriol-16-glucosiduronate in the pregnant human(5). There was little, if any, metabolism of the steroidal moiety. In the work described below, 17β-estradiol-6,7-^{3}H-17-glucosiduronate was injected into 2 females, in one case simultaneously with 17β-estradiol-4-^{14}C, and the urinary metabolites were investigated in some detail.

MATERIALS AND METHODS

Unlabelled steroids, reagents, etc.

17β-Estradiol-3-methyl ether was prepared in the laboratory by the method of Brown(6). All other steroids were purchased from Mann Research Labs. Inc., New York. All of the compounds were checked for purity by thin-layer chromatography and melting point (microscope hot stage) determination.

All organic solvents and reagents were of analytical grade and were purified where necessary by published methods (7,8).

Bacterial β-glucuronidase (Type II) was purchased from Sigma Chem. Co., St. Louis, Mo.

Radioactive compounds; their radiochemical purity

17β-Estradiol-4-^{14}C, specific activity (SA) = 0.17μc/μg was purchased from New England Nuclear Corp., Boston, Mass. and was found to be at least 98% pure by crystallizing with unlabelled 17β-estradiol. It was used without further purification.

17β-Estradiol-6,7-^{3}H-17-glucosiduronate, SA = 2.2μc/μg, was also purchased from the above source. Approximately 1% of this was ether-extractable from aqueous solution. The water-soluble portion was completely hydrolyzed by incubation with bacterial β-glucuronidase (9) and the ether-soluble radioactivity was shown to be at least 96% composed of 17β-estradiol by crystallization with carrier. 384,000 Dpm (disintegrations per minute) of the original labelled conjugate were methylated by the method of Brown (6). The product was incubated with β-glucuronidase as above and the ether-soluble radioactivity (377,300 dpm) was extracted. A portion of this (137,200 dpm) was mixed with 13.7 mg of pure unlabelled 17β-estradiol-3-methyl ether to give a calculated SA of 10,000 dpm/mg. Crystallization from methanol-H_2O yielded crystals of SA = 9674 dpm/mg. The latter were acetylated and recrystallized from methanol-H_2O yielding crystals of SA = 8306 dpm/mg. This compared with a predicted SA of 8457 dpm/mg based on the SA of the unacetylated material from the first crystallization.

Injection of radioactive compounds

Two young females, both having normal cycles, were volunteers for this study. Labelled compounds were injected intravenously in 10 ml of 10% (v/v) ethanol in saline. In expt. 1, 55 x 10^6 dpm of 17β-estradiol-6,7-^{3}H-17-glucosiduronate were given, while in expt. 2, 22.2 x 10^6 dpm of the latter were injected together with 4.18 x 10^6 dpm of 17β-estradiol-4-^{14}C.

Urine collection

Following injection, urine was collected from each subject for 4 x 24 hr in polyethylene bottles without preservative. Urine was stored at -15°C until analysis

could be commenced.

Analytical procedure

In expt. 1 the first 2 days' urine were pooled and one-half of this was taken for analysis. In expt. 2 the entire 4-day pool was used. The untreated urine was extracted with ether to yield a 'free' (unconjugated) steroid fraction, and this was repeated following β-glucuronidase hydrolysis, to yield a 'glucosiduronate' (G) fraction. The residual urine was solvolyzed giving a 'sulfate' (S) fraction. These steps are described elsewhere (9, 10). The G and S fractions were purified and resolved into their component steroid fractions by Girard separation, various extraction procedures and Celite column partition chromatography (9, 10). The separated fractions were mixed with the appropriate carrier steroids and crystallized 3 times from methanol. At this stage the SA values for crystals and mother liquors agreed for most fractions. The third crystals were acetylated at room temperature in a mixture of pyridine and acetic anhydride and recrystallized from methanol-H_2O. The SA values for the acetates were used to confirm the data obtained from the free steroid crystallizations. Melting points of the acetates were checked in order to confirm purity and derivative formation.

Measurement of radioactivity

All counting was performed on a Nuclear Chicago Liquid Scintillation Spectrometer. Urine was counted directly in a dioxan scintillation medium (9). All extracts, crystals, etc. were counted following solution in 0.5 ml of methanol and dilution with 10 ml of toluene containing 7 g/liter of butyl PBD [butyl derivative of 2-phenyl-5-(4-biphenylyl)-oxadiazole-1,3,4]. Quenching was corrected for, where necessary, by the internal standard technique. Calculation of ^3H and ^{14}C in dual-labelled samples was performed by the method of Okita et al (11). All counts were converted to dpm and sufficient counts were accumulated to ensure a counting error of no more than ±5%.

RESULTS

Table I contains evidence for rapid urinary excretion

TABLE I

Excretion and extraction of urinary radioactivity in expts. 1 and 2

Days following injection	Expt. 1 ^{3}H	Expt. 2 ^{3}H	Expt. 2 ^{14}C
	Urine radioactivity (% dose)		
1	73	58	37
2	12	13	16
3	3	0.8	6
4	1	0.3	4
Total	89	72	63
	dpm x 10^{-6}		
Urine pool	46.7(2 days)	15.8	2.63(4 days)
Free-ether soluble	0.58	0.16	0.046
G fraction	40.2	12.6	1.06
S fraction	2.8	1.6	0.26

of the ^{3}H label, primarily in days 1 and 2 following injection. The excretion of ^{14}C in expt. 2 was slower, 10% of the dose appearing on days 3 and 4. Table I also shows that, in expt. 1, 1% of the ^{3}H in the 48 hr urine pool was ether-extractable, 86% was hydrolyzed by β-glucuronidase and 6% was solvolyzable. In the 4-day pool from expt. 2, 1% of the ^{3}H was 'free,' 80% was in the G fraction and 11% was S, while for ^{14}C the corresponding values were 2% free, 40% G and 10% S. The $^{3}H/^{14}C$ ratios were; urine pool = 6.0, 'free' = 3.5, G = 12.0, S = 7.1.

Table II shows the results of crystallizing the various steroid fractions in expt. 1. The main metabolites, estrone

G and 17β-estradiol G, were calculated, from these data, to be 94% and 90% pure, respectively, as eluted from the partition columns. The other fractions were found to be of variable purities prior to crystallization.

Table III contains information regarding the $^{3}H/^{14}C$ ratios during crystallization of the metabolites isolated in expt. 2. Individual SA values are not shown since those with respect to ^{3}H were similar to those of expt. 1 (Table I) and since the purification of urinary metabolites of injected free 17β-estradiol has been the subject of much previous work (9, 12). As in expt. 1 SA values for crystals agreed well in most instances with those of the mother liquors at the third crystallization of the free steroids. The 16-oxygenated metabolites, whether conjugated as G or S, exhibited a lower isotope ratio than that of 5.3 which was injected, indicating a lesser production from the injected glucosiduronate than from free 17β-estradiol. Conversely, urinary estrone G, 17β-estradiol G and 17β-estradiol S were derived to a much greater extent from the injected conjugate than from the free steroid. This was also true, to a lesser extent, of 2-methoxyestrone G. 2-Methoxyestrone S and estrone S, on the other hand, possessed similar ratios to that injected.

TABLE II

Crystallization of ^{3}H-labelled metabolites in expt. 1.

Steroid fraction	Calculated SA(dpm/mg)*	Free steroids**				Acetatest	
		XLS 1	XLS 2	XLS 3	ML 3	XLS 4	XLS 5
2MeO-E_1 G	9,170	5,600	5,050	5,130	6,200	5,080	-
" S	3,220	1,820	1,560	1,600	2,210	1,305	1220
E_1 G	29,100	27,400	27,000	26,700	26,760	27,200	-
" S	8,640	6,670	6,330	6,215	6,600	6,150	-
E_2 G	22,200	19,920	19,370	19,240	20,260	20,300	-
" S	10,850	9,330	9,190	9,270	9,360	9,450	-
E_3 G	23,400	16,060	15,540	15,520	17,000	16,090	-
" S	1,185	767	-	780	873	810	822
16epiE_3 G	18,820	7,500	6,490	6,380	6,800	6,100	-
" S	1,280	758	737	741	800	720	720
16αOHE_1 G	9,145	6,910	6,680	6,810	7,150	7,100	-
" S	1,060	854	856	824	800	810	-
16KE_2 G	10,120	6,700	6,240	6,280	6,500	6,100	-
" S	2,270	1,990	1,790	1,890	1,890	1,860	-

*Calculated value based on wt. of carrier added to an aliquot of each fraction

**XLS = crystals; ML = mother liquor.

†Values shown have been calculated back to the free steroids.

TABLE III

$^{3}H/^{14}C$ Ratios of fractions during purification in expt. 2 (injected ratio = 5.3)

Steroid fraction	$^{3}H/^{14}C$ ratios						
	Free steroids*					Acetates	
	Column fractions	XLS 1	XLS 2	XLS 3	ML 3	XLS 4	XLS 5
2MeO-E_1 G	12.7	13.3	12.7	13.3	12.8	13.1	12.9
" S	5.6	5.8	5.4	5.3	5.9	5.3	5.3
E_1 G	26	29	29	30	28	29	28
" S	5.8	6.0	5.9	5.7	5.5	5.9	6.0
E_2 G	70	82	80	84	83	77	77
" S	52	60	62	61	61	61	60
E_3 G	2.7	2.0	1.9	-	2.0	1.8	1.8
" S	3.3	2.0	2.0	2.0	2.0	1.9	2.0
16epiE_3 G	7.0	2.8	2.2	2.1	2.3	2.0	1.8
" S	3.5	2.5	2.8	2.2	2.2	2.0	2.0
16αOHE_1 G	3.3	3.0	3.1	3.1	3.0	3.1	3.0
" S	2.2	1.7	1.8	2.0	1.9	1.9	-
16KE_2 G	3.1	2.7	2.7	2.8	2.9	2.7	2.7
" S	2.6	1.8	2.0	1.8	1.9	1.8	1.6

*XLS = crystals; ML = mother liquor.

Table IV shows the amounts of radioactivity associated with the purified steroid fractions isolated in the 2 expts. These values, uncorrected for methodological loss, were obtained from the mathematical product of the SA of the purified crystals and the weight of carrier steroid used in each particular case. More than 80% of the identified ^{3}H-labelled metabolites consisted of estrone G and 17β-estradiol G whereas only 3% was attributable to estriol G. Of the ^{14}C-labelled metabolites identified in expt. 2, estrone G and 17β-estradiol G together comprised 33% and estriol G accounted for 27%. As a group, the 16-oxygenated metabolites of 17β-estradiol-6,7-^{3}H-17-glucosiduronate were of minor significance. In both expts. ^{3}H-labelled 17β-estradiol S, although not by any means a major metabolite, exceeded ^{3}H-estrone S.

DISCUSSION

The data presented above demonstrate very clearly that the 17-glucosiduronate of 17β-estradiol can be metabolized in the human. The main urinary metabolites are estrone and 17β-estradiol, in a ratio of about unity, both apparently conjugated with glucuronic acid. It is not known at present how this conversion proceeds but, in view of the considerably greater production of urinary estrone G from injected 17β-estradiol glucosiduronate than

TABLE IV

Relative amounts of labelled metabolites following crystallization in expts. 1 and 2

Steroid fraction	Expt. 1 (2 days)		Expt. 2 (4 days)			
	^{3}H (dpm x 10^{-5})	% of total identified	^{3}H (dpm x 10^{-5})	% of total identified	^{14}C (dpm x 10^{-4})	% of total identified
2MeO-E_1 G	2.3	<1	1.4	2	1.1	2
" S	0.5	<1	0.4	<1	0.8	<2
E_1 G	139	48	31	38	11.0	23
" S	3.3	1	0.7	<1	1.2	3
E_2 G	121	41	37	45	4.7	10
" S	5.3	2	4.7	6	0.8	<2
E_3 G	9.4	3	2.4	3	12.8	27
" S	0.4	<1	0.1	<1	0.7	<2
16epiE_3 G	2.7	<1	0.6	<1	3.0	6
" S	0.4	<1	0.1	<1	0.4	<1
16αOHE_1 G	4.2	1	2.2	3	7.0	15
" S	0.4	<1	0.2	<1	1.0	2
16KE_2 G	2.4	<1	0.8	<1	3.0	6
" S	0.9	<1	0.2	<1	0.9	<2
Total	292		82		48	

from 17β-estradiol, it is conceivable that the administered steroid glucosiduronate could have been further conjugated (e.g. to form the diglucosiduronate) prior to removal of the glucuronic acid at C_{17} to yield estrone glucosiduronate, presumably via 17β-estradiol-3-glucosiduronate. Such a metabolic pathway could perhaps explain the very low production of 16-oxygenated metabolites from 17β-estradiol glucosiduronate. It is of interest to note, in passing, that ^{3}H-17β-estradiol S exceeded, in both expts., ^{3}H-estrone S. This differs from the pattern obtained for the metabolism of injected 17β-estradiol(9). It raises the question as to whether the injected 17β-estradiol-6,7-^{3}H-17-glucosiduronate might have been partly sulfurylated at position 3 to yield a mixed conjugate. It must be borne in mind that our identification of 17β-estradiol S was made following serial β-glucuronidase incubation and solvolysis, thus it does not exclude the presence of a monosulfate, a disulfate or a mixed conjugate, or any combination of these.

The only urinary steroid fractions possessing $^{3}H/^{14}C$ ratios similar to that injected were 2-methoxyestrone S and estrone S, presumably representing the 3-sulfates of these steroids. The relationship between the ratios of these fractions, the high isotope ratios

of the other 16-desoxy-metabolites and the low ratios of the 16-oxygenated steroids is not at all clear at present. Further speculation would be unjustified at this time since it is obvious that answers to many of the questions posed above must await studies involving direct separation and purification of individual conjugates.

It can be seen from the data presented in this paper that a considerably greater fraction of the metabolites of injected 17β-estradiol glucosiduronate were extracted from the urine and identified than is generally true of metabolites of 17β-estradiol (expt. 2, above, and ref. 9). The presence of the glucuronic acid residue presumably decreases the degree of metabolism (e.g. as reflected in the small extent of 16-hydroxylation) with an accompanying decrease in the amount of unknown and less stable conversion products.

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2. Medical Research Associate of The Medical Research Council of Canada.

3. The following trivial names and abbreviations for steroids are employed in this paper:
2-Methoxyestrone (2MeO-E_1) = 2-methoxy-3-hydroxyestra-1,3,5(10)-trien-17-one
Estrone (E_1) = 3-hydroxyestra-1,3,5(10)-trien-17-one
17β-Estradiol (E_2) = estra-1,3,5(10)-triene-3,17β-diol
Estriol (E_3) = estra-1,3,5(10)-triene-3,16α,17β-triol
16α-Hydroxyestrone (16αOHE_1) = 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one
16-Ketoestradiol-17β (16KE_2) = 3,17β-dihydroxyestra-1,3,5(10)-trien-16-one
16-Epiestriol (16epiE_3) = estra-1,3,5(10)-triene-3,16β,17β-triol
Other abbreviations used: G = steroid fraction rendered ether-soluble by incubation with β-glucuronidase; S = solvolyzable steroid conjugate fraction remaining following initial β-glucuronidase hydrolysis, and ether-extraction of the steroids so released.

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IDENTIFICATION OF SOME URINARY CONJUGATED METABOLITES OF 17β-ESTRADIOL-6,7-^{3}H-17-GLUCOSIDURONATE IN THE HUMAN FEMALE (1)

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ABSTRACT

Following intravenous injection of 17β-estradiol-6,7-^{3}H-17-glucosiduronate into a normal human female the urinary conjugated forms of labelled estrone and 17β-estradiol were identified by DEAE-Sephadex and Celite partition chromatography; comparison with certain authentic ^{14}C-labelled conjugates; enzyme hydrolysis; crystallization with carrier steroids, including derivative formation. The main compounds identified were estrone-3-glucosiduronate, 17β-estradiol-17-glucosiduronate and 17β-estradiol-3-glucosiduronate in the approximate proportions of 5.6:3.7:1. Some evidence was also obtained for the presence of very small amounts of estrone-3-sulfate and 17β-estradiol-3-sulfate-17-glucosiduronate.

INTRODUCTION

It was recently shown (3) that intravenously injected 17β-estradiol-6,7-^{3}H-17-glucosiduronate ($E_2$17G; reference 4) in normal human females resulted in the urinary excretion of approximately equal amounts of estrone (E_1) and 17β-estradiol (E_2), both mainly in a form hydrolyzable by β-glucuronidase. Very small amounts of 16-oxygenated metabolites were found, suggesting interference with 16-hydroxylation by the glucuronic acid residue. Since, in these experiments, identification of the steroid metabolites was entirely based

on purification following conjugate hydrolysis, it was considered necessary to establish more directly the identities of the specific conjugates. The present paper deals with this identification.

MATERIALS

Amberlite XAD-2 resin was purchased from Rohm and Haas, Philadelphia, Pa. DEAE-Sephadex (A-25) was purchased from Pharmacia (Canada) Ltd. and was used for chromatography (column dimensions 0.9 x 50 cm) as described earlier (5). Celite was treated by conventional methods prior to use (6). Silica gel HF_{254}, for thin-layer chromatography (TLC), was a product of Merck, Darmstadt, Germany, and was spread on 20 x 20 cm glass plates prior to activating at 110° for 1 hr. All organic solvents and chemical reagents were of good analytical grade and were purified, where necessary, by standard methods (6,7).

Unlabelled steroids and their derivatives were purchased from Mann Research Labs., New York, and were checked for purity by TLC and melting point (microscope hot stage) determination. 17β-Estradiol-3-methyl ether (E_2 3MeE) was crystallized x 2 from methanol-H_2O prior to use.

Mylase P, containing phenolsulfatase, was obtained from Mann Research Labs., New York, and was used for conjugate hydrolysis in 0.1M acetate buffer, pH 6, overnight (8). Bacterial β-glucuronidase powder (Type II) was obtained from Sigma Chemical Co., St. Louis, Mo. It was used in 0.1M phosphate buffer, pH 7, at a concentration of 20 - 40 units/ml for 24 hr (9).

Estrone-4-^{14}C-3-glucosiduronate (E_1 3G) was synthesized by incubating pure E_1-4-^{14}C, specific activity (SA) = 0.1μc/μg (Radiochemical Centre, Amersham, Bucks, England) with a rabbit liver homogenate as described earlier (5). The ^{14}C-E_1 3G was purified by DEAE-Sephadex and Celite partition chromatography (5). Following β-glucuronidase hydrolysis, at least 97% of the ^{14}C-steroid released was shown (by crystallization with carrier) to be E_1. 17β-Estradiol-4-^{14}C-3-glucosiduronate (E_2 3G) was prepared from the above ^{14}C-E_1 3G by reduction with $NaBH_4$ (5). The reduced material was purified as for ^{14}C-E_1 3G and shown to yield E_2 (at least 97% pure) on β-glucuronidase hydrolysis.

^{3}H-E_2 17G (SA = 2.2 μc/μg) was purchased from New England Nuclear Corp., Boston, Mass. and was found to behave in a homogeneous fashion on DEAE-Sephadex and on Celite partition columns (5). It was 97% pure by crystallization procedures already described (3).

Urine was collected for 2 successive 24 hr periods (pooled prior to analysis) from a normal young female who had been injected intravenously with 55 x 10^6 dpm (disintegrations per minute) of ^{3}H-E_2 17G (3). Some 85% of the dose was excreted in 48 hr.

METHODS

Whole urine was processed on Amberlite XAD-2 as described by Bradlow (10). The conjugate fraction so obtained was chromatographed on DEAE-Sephadex in a linear gradient of 0 - 0.8M NaCl (5) and radioactive peaks corresponding to monoglucosiduronates were successively re-chromatographed on DEAE-Sephadex in a non-linear gradient 0 - 1M NaCl (11) and a linear gradient 0 - 0.4M NaCl (5). In all cases the eluates were processed on XAD-2 resin in order to recover the conjugates in methanol.

The various monoglucosiduronate peaks, and radioactivity corresponding to monosulfate(s), were chromatographed on Celite (1 x 20 cm column) in iso-octane:t-butanol:MNH_4OH (2:5:5; system A; ref. 12). Radioactivity corresponding to mixed, or double conjugate(s) was chromatographed similarly on Celite in n-butanol:ethyl acetate:0.2% NH_4OH (3:1:4; system B; ref. 13).

Aliquots of individual ^{3}H-monoglucosiduronate fractions, with or without addition of appropriate ^{14}C-conjugates, were identified by further Celite chromatography in the conjugated form, β-glucuronidase hydrolysis (9), Girard separation (14), Celite chromatography of the free steroids (14) and by crystallization to constant SA or ^{3}H/^{14}C ratio with unlabelled carrier steroids (including derivative formation). Further aliquots of glucosiduronate fractions were methylated by the procedure of Brown (15) to localize a free phenolic group. Following hydrolysis (3) the resulting ether-soluble radioactivity was submitted to TLC in cyclohexane:ethyl acetate (1:1; ref. 16) in admixture with ca. 300 μg of the appropriate standard steroids. Appropriate ultraviolet-absorbing zones were eluted with ethyl acetate:methanol (1:1)

and crystallized with carrier steroids. A presumptive ^{3}H-E_2 3G fraction was methylated with methyl iodide by the procedure described by Sa'at & Slaunwhite (17) to localize the non-phenolic hydroxyl group. After hydrolysis, the steroidal moiety was successively chromatographed on Celite in chloroform:carbon tetrachloride:methanol:H_2O (40:60:70:30; system C; ref. 17) and benzene:0.8N NaOH (system D; ref. 18).

Presumptive ^{3}H-monosulfate(s) was hydrolyzed with Mylase P and the ether-soluble material submitted to a Girard separation followed by crystallization with carrier steroid.

An aliquot of presumptive ^{3}H-mixed conjugate(s) was incubated with Mylase P (followed by ether extraction) and the aqueous residue with β-glucuronidase (followed by ether extraction). A further aliquot was treated in the reverse manner. Ether-soluble radioactivity was submitted to a Girard separation and then to crystallization with carrier steroid.

All final crystalline derivatives were submitted to melting point determination.

All radioactive counting was performed on a Nuclear Chicago Liquid Scintillation Spectrometer as described elsewhere (3, 9). The counting error was within ±5%.

RESULTS

Preliminary separation

Fig. 1 shows the pattern of radioactivity obtained on DEAE-Sephadex chromatography of the urinary conjugates (one-sixth of the 2-day pool). Peak 1 (fractions 14 - 30) resembled E_1 3G together with some E_2 3G (5) and peak 2 (31 - 35) behaved like E_2 17G (5). Peaks 3 (38 - 54) and 4 (68 - 77) were poorly defined but resembled monosulfate(s) and mixed conjugate(s), respectively (5). Further elution with 0.8 - 2M NaCl (5) failed to show the presence of disulfate

metabolites. In the non-linear gradient, peak 1 was resolved into 1A (61 - 70; resembling E_1 3G in behaviour) and 1B (73 - 80; resembling E_2 3G) as shown in Fig. 2. Peak 2 behaved as a single peak (79 - 95) in the latter gradient (Fig. 3).

Identity of peak 1A

Some 88% of the ^3H in this peak was eluted from Celite (system A) at about holdback volume (HBV) 10, most of the remainder (not further processed) being eluted with methanol. An aliquot of the apparent ^3H-E_1 3G was completely hydrolyzed by β-glucuronidase and the ether-soluble ^3H shown to be >90% ketonic. Preliminary crystallization showed the latter to be predominantly E_1. A second aliquot of the conjugate fraction was eluted from DEAE-Sephadex (0 - 0.4M NaCl) as a symmetrical peak at ca. 0.14M NaCl and was separable from ^{14}C-E_2 3G (peak at 0.16M NaCl) and ^3H-E_2 17G (peak at 0.18M NaCl) when chromatographed in admixture with them in the same gradient. A third aliquot containing 1.65 x 10^6 dpm ^3H was mixed with 0.262 x 10^6 dpm ^{14}C-E_1 3G ($^3H/^{14}C$ = 6.3) and re-chromatographed on Celite (system A). The peak tubes were pooled giving 1.34 x 10^6 dpm ^3H and 0.222 x 10^6 dpm ^{14}C ($^3H/^{14}C$ = 6.0). This material was completely hydrolyzed by β-glucuronidase and an aliquot was mixed with unlabelled E_1 and crystallized as shown in Table I. This indicated a high

proportion of the ^{3}H in peak 1A to be identifiable with E_1 3G.

TABLE I

Crystallization of $^{3}H/^{14}C$-E_1 from peak 1A

Purification procedure*	SA(^{3}H) (dpm/mg)	SA(^{14}C) (dpm/mg)	$^{3}H/^{14}C$
402,700 Dpm ^{3}H + 6,700 dpm ^{14}C + 25.5 mg carrier E_1 ; crystn. from methanol	15,800 (calc.)	2,630 (calc.)	6.0
XLS 1	16,000	2,690	6.0
ML 1	17,300	2,730	
XLS 2	15,900	2,610	6.1
ML 2	16,100	2,860	
Acetylation of XLS 2; crystn. from methanol-H_2O	13,800 (calc.)	2,260 (calc.)	
XLS 3	13,500	2,240	6.0
ML 3	14,000	2,450	
XLS 4	13,700	2,250	6.1
ML 4	13,800	2,310	

*XLS = crystals; ML = mother liquor.

Identity of peak 1B

Celite chromatography in system A resulted in elution of a main peak (HBV 9; 83% of recovered ^{3}H) with minor amounts at HBV 14 (12%) and on elution with methanol (5%). The two latter were not further analyzed. An aliquot of the main peak was completely hydrolyzed by β-glucuronidase, the free material being ca. 90% non-ketonic and on preliminary crystallization behaving like E_2. Following mild methylation (15) and hydrolysis of a further aliquot of 1B, TLC of the products showed that ca. 40% of recovered ^{3}H had the

mobility of E_2 and ca. 60% was similar to E_2 3MeE. Crystallization of these TLC zones with carriers gave the results shown in Table II. These data strongly suggested that peak 1B was largely composed of a mixture of a monoglucosiduronate of E_2 lacking a free phenolic group (E_2 3G) and another such compound possessing a free phenolic group (E_2 17G). The presence of the latter was presumably due to contamination by peak 2. A further 1B peak was prepared from a separate aliquot of urine exactly as before, except that 5 ml fractions were collected from the 0 - 0.8M NaCl gradient, and care was taken to exclude, as far as possible, contamination by peak 2 during pooling of chromatographic fractions. This 1B peak yielded two peaks on rechromatography in 0 - 0.4M NaCl (Fig. 4). Fractions 57 - 63, when pooled as peak 1B-(1), contained 538,000 dpm ^3H, while fractions 64 - 70, peak 1B-(2), contained 208,000 dpm.

Identity of peak 1B-(1)

290,000 Dpm ^3H from peak 1B-(1), mixed with 58,600 dpm ^{14}C-E_2 3G ($^3H/^{14}C$ = 4.9) were eluted from Celite (system A) at about HBV 9 with an isotope ratio = 4.5. Mild methylation (15) of an aliquot, followed by hydrolysis and TLC of the products, showed that only 3% of the recovered ^3H and 2% of the ^{14}C possessed the mobility of E_2 3MeE whereas almost all of the remainder was associated with carrier E_2.

The results of crystallization of the latter with E_2 (Table III), together with the data presented above, strongly indicated that peak 1B-(1) consisted largely of a mono-glucosiduronate of E_2 lacking a free phenolic group, i.e. E_2 3G. The remainder of peak 1B-(1), mixed with ^{14}C-E_2 3G

TABLE III

Crystallization of $^{3}H/^{14}C$-E_2 from peak 1B-(1)

Purification procedure*	SA(^{3}H)	SA(^{14}C) (dpm/mg)	$^{3}H/^{14}C$
83,500 Dpm ^{3}H + 20,900 dpm ^{14}C from TLC + 26.6 mg E_2; crystn. from methanol	3140 (calc.)	786 (calc.)	4.0
XLS 1	3140	766	4.1
ML 1	3160	771	
XLS 2	3010	753	4.0
ML 2	3100	775	
Acetylation of XLS 2; crystn. from methanol-H_2O	2300 (calc.)	576 (calc.)	
XLS 3	2460	598	4.1
ML 3	2410	610	
XLS 4	2380	604	3.9
ML 4	2450	594	

*XLS = crystals; ML = mother liquor.

($^{3}H/^{14}C$ = 5.0) and chromatographed on Celite in system A ($^{3}H/^{14}C$ = 4.3), gave, on forced methylation (17) a hexane-soluble material, $^{3}H/^{14}C$ = 4.4. Following hydrolysis the ratio was 4.0. The labelled product was successively eluted as a single peak of low polarity (considerably less polar than E_2) from Celite in systems C and D with an isotope

TABLE II

Crystallization of ^{3}H-E_2 and ^{3}H-E_2 3MeE from peak 1B

E_2		E_2 3MeE	
Purification procedure*	SA(dpm/mg)	Purification procedure*	SA(dpm/mg)
18,300 Dpm ^{3}H from TLC + 28.8 mg E_2; crystn. from methanol	635 (calc.)	28,000 Dpm ^{3}H from TLC + 25.7 mg E_2 3MeE; crystn. from methanol-H_2O	1090 (calc.)
XLS 1	561	XLS 1	1010
ML 1	641	ML 1	1123
XLS 2	596	XLS 2	1010
ML 2	625	ML 2	1067
Acetylation of XLS 2; crystn. from methanol-H_2O	455 (calc.)	Acetylation of XLS 2; crystn. from methanol-H_2O	880 (calc.)
XLS 3	453	XLS 3	900
ML 3	557	ML 3	910
XLS 4	461	XLS 4	870
ML 4	†	ML 4	890

*XLS = crystals; ML = mother liquor
†Insufficient for counting.

ratio of 4.2. It compared in mobility with 17β-estradiol-17-methyl ether.

Identity of peak 1B-(2)

Chromatography on Celite in system A yielded a peak corresponding to a monoglucosiduronate. Hydrolysis with β-glucuronidase was complete and some 94% of the ether-soluble radioactivity was non-ketonic. Celite partition chromatography in the system benzene:hexane:methanol:H_2O (55:45:70:30; ref. 14) followed by benzene:methanol:H_2O (100:70:30; ref. 14) yielded almost 90% recovery of the ^3H of which 93% corresponded to E_2, 3% to 16-epiestriol and 4% to estriol. It was logical to assign the identity of $E_2$17G (originally contaminating $E_2$3G) to this minor peak.

Identity of peak 2

A single peak was eluted at HBV 9 (monoglucosiduronate region) from Celite in system **A.** An aliquot was completely hydrolyzed by β-glucuronidase, the ether-soluble material being some 92% non-ketonic. The latter was shown to be mainly E_2 by preliminary crystallization. Mild methylation (15) of an aliquot followed by hydrolysis and TLC resulted in <2% of the recovered ^3H being associated with carrier E_2. Virtually all of the remainder had the mobility of $E_2$3MeE. Crystallization with carrier gave the results shown in Table IV. **A** further aliquot of 2 was eluted from DEAE-Sephadex

TABLE IV

Crystallization of ^{3}H-E_2 3MeE from peak 2

Purification procedure*	SA(dpm/mg)
64,500 Dpm ^{3}H from TLC	2590 (calc.)
+ 24.9 mg E_2 3MeE; crystn. from methanol-H_2O	
XLS 1	2460
ML 1	2500
XLS 2	2480
ML 2	2550
Acetylation of XLS 2; crystn. from methanol-H_2O	2160 (calc.)
XLS 3	2280
ML 3	†
XLS 4	2220
ML 4	2260

*XLS = crystals; ML = mother liquor.
†Insufficient for counting.

(0 - 0.4M NaCl) as a single symmetrical peak at 0.18M NaCl and separable from simultaneously chromatographed ^{14}C-E_2 3G. These data identified peak 2 as being mainly composed of E_2 17G.

Identity of peak 3

Radioactivity isolated from separate 2 x one-sixth aliquots of the 48 hr urine, and pooled to yield sufficient ^{3}H for analysis, when chromatographed on Celite in system A yielded a well-defined peak (50% of the applied ^{3}H) at HBV 2 - 3 (monosulfate region). There was also a poorly defined elution of ^{3}H at HBV 7; this was not processed. 136,000 Dpm

from the first peak were hydrolyzed to an extent of 75% by Mylase P; the ether-soluble ^{3}H shown to be 92% ketonic. 94,000 Dpm of the latter were mixed with 17.9 mg carrier E_1 (calc. SA = 5250 dpm/mg) and crystallized twice from methanol. XLS of SA = 4370 and 4090 and MLS of 5410 and 5090, respectively, were obtained. The second XLS were acetylated (calc. SA = 3540 dpm/mg) and crystallized twice from methanol-H_2O giving XLS of SA = 3420 and 3440 and MLS of 3770 and 3820, respectively. This suggested that estrone-3-sulfate (E_1 3S) was a component of peak 3.

Identity of peak 4

Radioactivity isolated from 2 x one-sixth aliquots of the 48 hr urine pool, when chromatographed on Celite in system B, resulted in elution of 54% of the applied ^{3}H at HBV 4 (i.e. typical of a compound such as 17β-estradiol-3-sulfate-17-glucosiduronate; E_2 3S17G; ref. 5). The remainder of the ^{3}H was not investigated. Two aliquots, each containing 44,000 dpm, when submitted to enzyme hydrolysis, showed some 5% of the ^{3}H to be ether-extractable after Mylase P alone and none following β-glucuronidase alone. When each of the partially hydrolyzed fractions was then incubated with the other enzyme, i.e. Mylase P followed by β-glucuronidase, and the reverse, complete hydrolysis was achieved and 96% of the ether-soluble product was non-

ketonic. 83,600 Dpm of the latter were mixed with 27.5 mg carrier E_2 (Calc. SA = 3040 dpm/mg) and crystallized twice from methanol, whereupon XLS of SA = 2880 and 2870 and MLS of 3150 and 3000 were obtained. Acetylation of the second XLS (calc. SA = 2190 dpm/mg) followed by two crystallizations from methanol-H_2O yielded XLS of SA = 2200 and 2130 and MLS of 2160 and 2300, respectively. These data supported the presence of a mixed conjugate of E_2, probably E_2 3S17G, in peak 4.

Relative amounts of conjugated metabolites

Following the purification steps described above, the amounts of ^3H identified in the form of E_1 3G, E_2 17G and E_2 3G in one-sixth of the 48 hr urine were 1.89, 1.26 and 0.34 (all x 10^6 dpm), respectively; i.e. the three compounds were present in the ratio, 5.6:3.7:1. This ratio should be considered approximate only since no account was taken of losses associated with the procedures employed and, on occasion, following chromatography, peak tubes only were pooled to afford a better opportunity of isolating each compound in its pure form. Thus the 'real total' for each conjugate is not known. Using these latter data the E_1 + E_2 monoglucosiduronate fraction can be calculated to be composed 54% of E_1 3G, 36% of E_2 17G and 10% of E_2 3G (approx.). The E_1 3S and E_2 3S17G fractions each accounted for about 1%

of the total E_1 + E_2 conjugated urinary fraction.

DISCUSSION

Earlier studies have shown that about 85% of identified urinary metabolites of injected ^{3}H-E_2 17G in the human female consisted of E_1 and E_2 conjugated in a form hydrolyzable by β-glucuronidase (3). The results of the present work thus identify the major metabolite of E_2 17G as being E_1 3G. The sequence of steps leading to the latter compound is not clear. Urinary E_2 17G is identified as the conjugate second in quantitative importance to E_1 3G. It may represent unchanged injected E_2 17G but this will not be ascertained until studies can be undertaken with doubly labelled conjugates. The smaller amount of E_2 3G identified could represent, wholly or partly, an intermediate in the conversion of E_2 17G to E_1 3G. It could also conceivably have arisen by metabolic reduction of the latter metabolite. It would certainly seem logical to suggest that E_2 3G is an intermediate in the conversion of E_2 17G to E_1 3G, perhaps via a diglucosiduronate form.

Sufficient evidence would appear to have been obtained for the presence of minor amounts of E_1 3S and E_2 3S17G in this study. Identification of the latter supports an earlier speculation that this mixed conjugate could be a metabolite of E_2 17G (3).

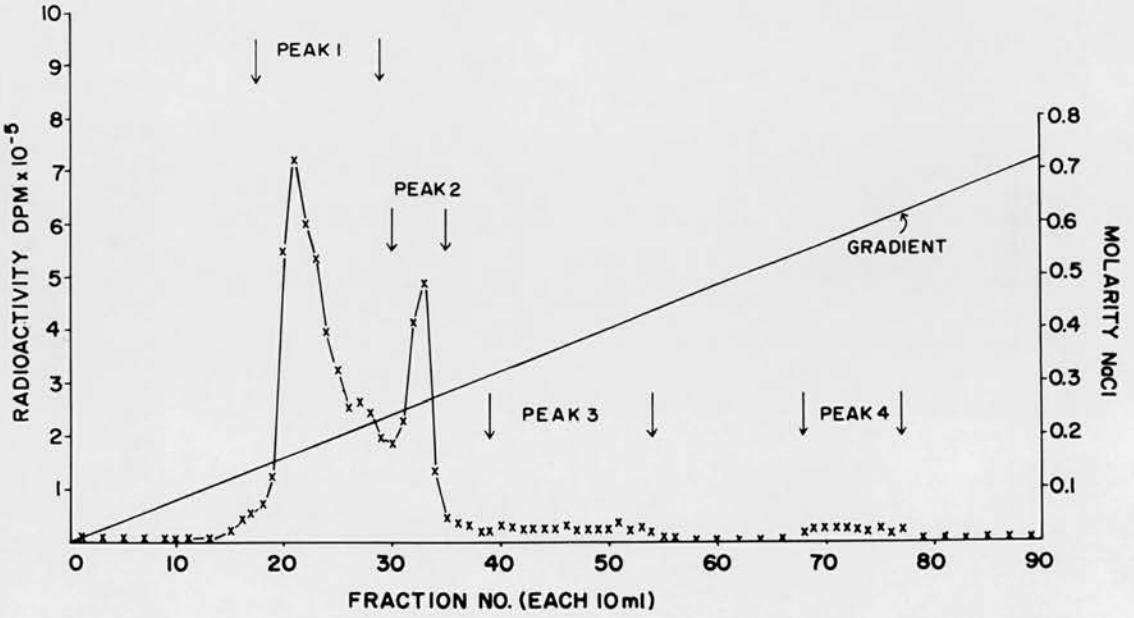

Fig. 1. DEAE-Sephadex chromatography (0 - 0.8M NaCl, linear gradient) of total urinary conjugate fraction from one-sixth of a 48 hr pool.

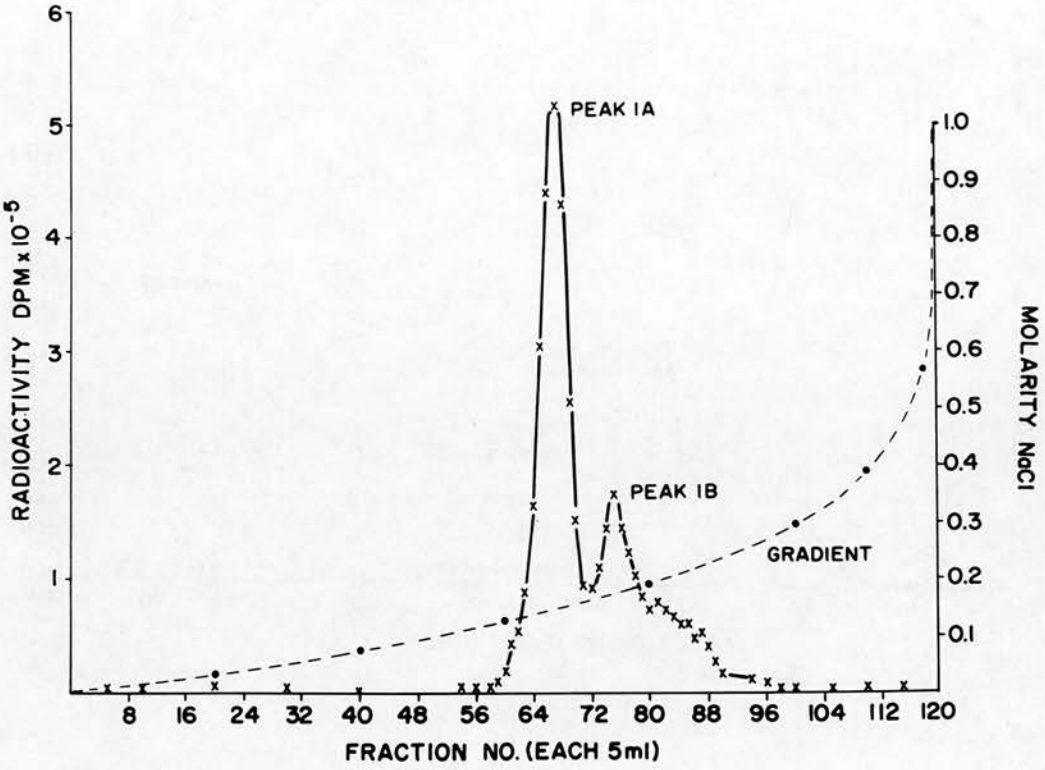

Fig. 2. Rechromatography of peak 1 on DEAE-Sephadex (0 - 1M NaCl, non-linear gradient).

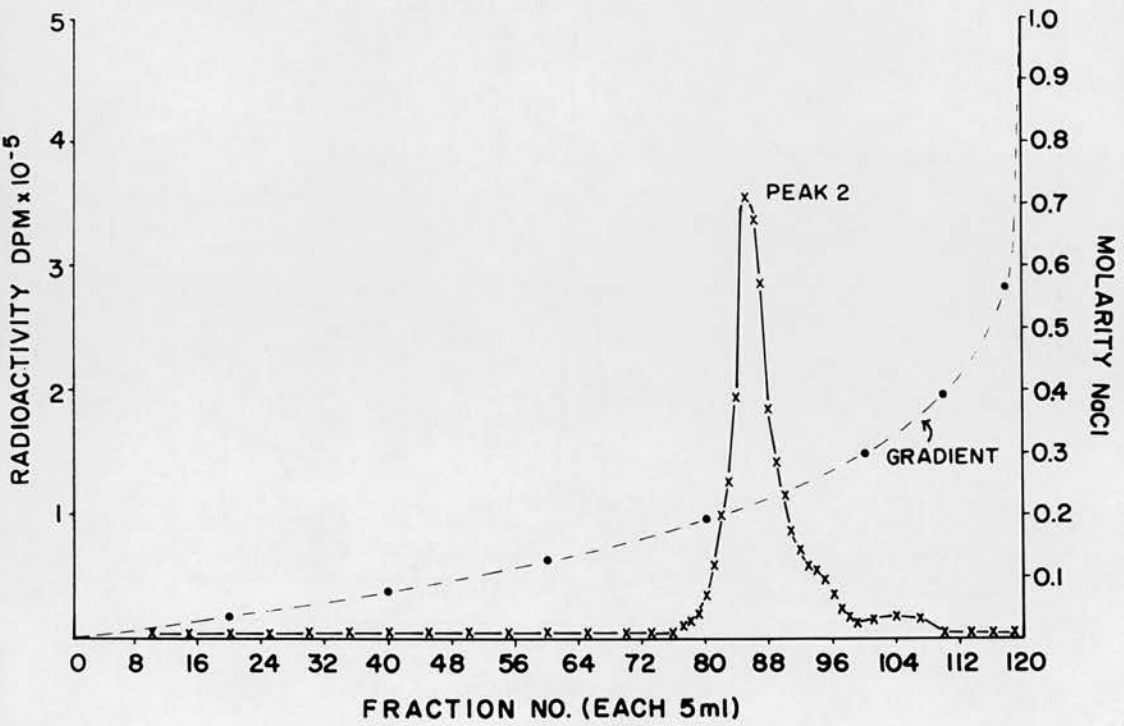

Fig. 3. Rechromatography of peak 2 on DEAE-Sephadex (0 - 1M NaCl, non-linear gradient).

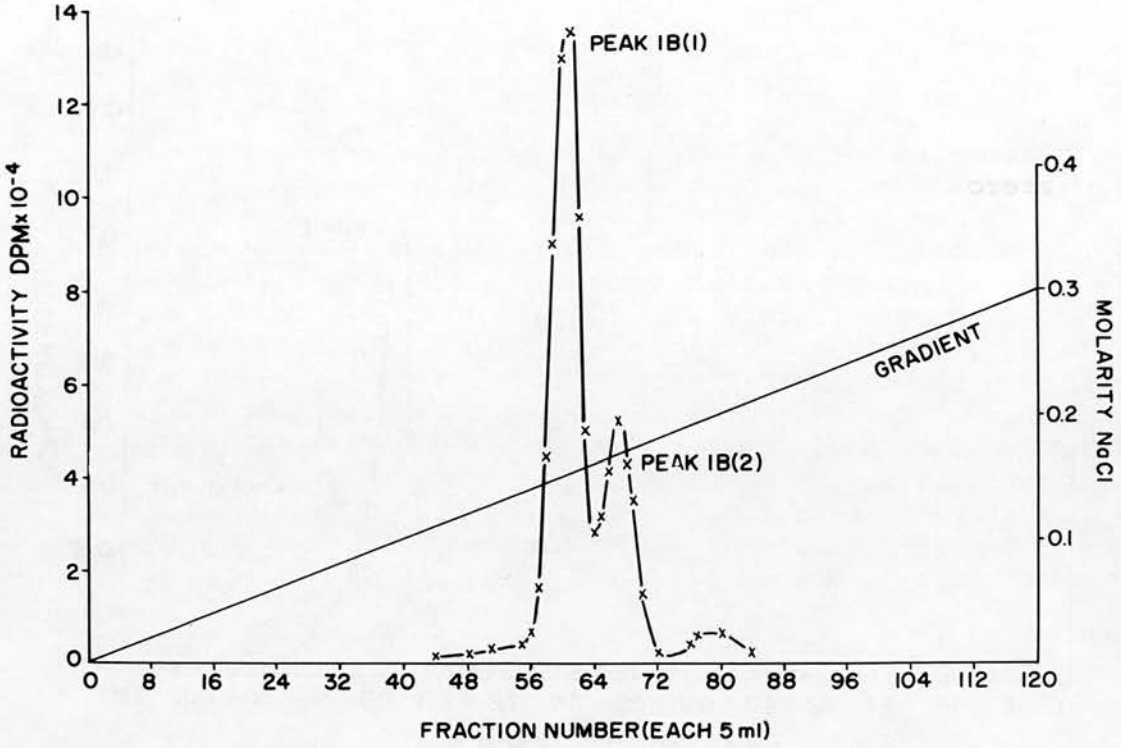

Fig. 4. Rechromatography of peak 1B on DEAE-Sephadex (0 - 0.4 M NaCl, linear gradient).

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We are indebted to Dr. P. R. Blahey, Dept. of Obstetrics and Gynecology, for his collaboration in this work.

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1. Supported by grant no. MT 532 from the Medical Research Council of Canada.

2. Medical Research Associate of the Medical Research Council of Canada.

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4. The following trivial names and abbreviations for steroids are employed in this paper: estrone (E_1) = 3-hydroxyestra-1,3,5(10)-trien-17-one; 17β-estradiol (E_2) = estra-1,3,5(10)-triene-3,17β-diol; estrone-3-glucosiduronate (E_1 3G) = 17-oxoestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-3-glucosiduronate (E_2 3G) = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-17-glucosiduronate (E_2 17G) = 3-hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate; estrone-3-sulfate (E_1 3S) = 17-oxoestra-1,3,5(10)-trien-3-yl-sulfate; 17β-estradiol-3-sulfate-17-glucosiduronate (E_2 3S17G) = 3-sulfo-estra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate; 17β-estradiol-3-methyl ether = E_2 3MeE.

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METABOLISM OF ESTRONE-3-GLUCOSIDURONATE AND 17β-ESTRADIOL-3-GLUCOSIDURONATE IN THE HUMAN FEMALE(1)

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ABSTRACT

Estrone-6,7-^{3}H-3-glucosiduronate-^{14}C (^{3}H-E_1 3G-^{14}C) and 17β-estradiol-6,7-^{3}H-3-glucosiduronate-^{14}C (^{3}H-E_2 3G-^{14}C), both of ^{3}H/^{14}C ratio = 5.8, were each injected into two normal young females. Seventy percent of each isotope was excreted in the urine in 3 hr, and at 48 hr, when excretion of the radioactivity had ceased, some 90% of ^{3}H and 75% of ^{14}C had been excreted. Urinary metabolites were purified and identified by Amberlite XAD-2 resin, DEAE-Sephadex and Celite partition chromatography followed by derivative formation, enzymic hydrolysis and crystallization with carrier steroids. No urinary metabolites other than E_1 3G and E_2 3G were identified. Within 3 hr of injection direct interconversion of E_1 3G and E_2 3G was observed as reflected in the ^{3}H/^{14}C ratios of the urinary metabolites which were the same as that injected. Under the experimental conditions the conversion, E_2 3G → E_1 3G, was much greater than the reverse. From 3 to 6 hr after injection the urinary metabolites possessed higher ^{3}H/^{14}C ratios (7.1 - 13.5) than that injected, indicating partial deconjugation to have taken place. From 6 to 24 hr after injection no ^{14}C could be detected with certainty to be associated with the ^{3}H-metabolites, showing that the latter had been produced through a process of initial deconjugation, followed by reconjugation with glucuronic acid of endogenous origin.

INTRODUCTION

Zucconi *et al* (3) reported an extremely rapid urinary excretion of radioactivity following intravenous infusion of

labelled estrone-3-glucosiduronate (E_1 3G; reference 4) into pregnant women. The results suggested little metabolism of the estrogen conjugate. The same authors (3), however, showed that the human fetus was capable of converting ^{3}H-E_1 3G-^{14}C (i.e. E_1 3G labelled with ^{3}H in the steroidal moiety and ^{14}C in the glucuronic acid residue) to ^{3}H-17β-estradiol-3-glucosiduronate-^{14}C (^{3}H-E_2 3G-^{14}C) of unchanged ^{3}H/^{14}C ratio, thus proving the occurrence of reduction of the C-17 keto group without removal of the glucosiduronate function. Moreover, it was indicated that 16α-hydroxylation of the conjugated steroid could also take place. More recently, Roy and Slaunwhite (5) reported upon the interconversion of E_1 3G and E_2 3G in the presence of a placental dehydrogenase enzyme together with suitable cofactors. These reactions proceeded without removal of the glucuronic acid residue. Finally, a recent speculation (6) regarding the metabolism of 17β-estradiol-17-glucosiduronate (E_2 17G) to E_1 3G depended upon the conversion of E_2 3G to E_1 3G in the form of an intact steroid conjugate.

The present paper deals with the fate of ^{3}H-E_1 3G-^{14}C and ^{3}H-E_2 3G-^{14}C when injected intravenously into normal women.

MATERIALS

General

Chromatographic materials, organic solvents and other reagents were obtained, purified when necessary, and used as described in earlier publications (6,7,8,9). Unlabelled steroids were purchased from Mann Research Labs., New York, and were checked for purity by melting point determination and thin-layer chromatography (TLC). Bacterial β-glucuronidase powder (Type II) was obtained from Sigma Chemical Co., St. Louis, Mo. and was used at pH 7 at a concentration of 40 units/ml for 24 hr at 38° C. Ketodase (beef liver β-glucuronidase) was purchased from Warner-Chilcott, Morris Plains, New Jersey, and was used for hydrolysis in amounts of 250 - 500 units/ml at pH 5 in 0.05M acetate buffer at 38° C with incubation times of 20 - 22 hr.

Estrone-6,7-^{3}H of specific activity (SA) = 48 mCi/μmole was purchased from New England Nuclear Corp., Boston, Mass., and was chromatographed on Celite prior to use (10). It was at least 98% pure by reverse isotope dilution with unlabelled estrone. Uridine diphosphate glucuronic acid (UDPGA)-^{14}C (glucuronic acid uniformly labelled) of SA = ca. 230 μCi/μmole was purchased from New England Nuclear Corporation.

Synthesis of ^{3}H-E_1 3G (see refs. 7, 11)

About 100 μCi of estrone-6,7-^{3}H were incubated for 1 hr at 37° C with a whole homogenate of female guinea pig liver in 0.1M phosphate buffer (400 mg liver/5 ml buffer) together with 1 mg of unlabelled UDPGA (Sigma Chem. Co.). Protein was precipitated with 4 vols of ethanol, the mixture cooled overnight at -15° C, and centrifuged. The supernatant was distributed between n-hexane and 90% methanol and the methanolic phase evaporated prior to chromatographing on DEAE-Sephadex in a linear 0 - 0.8M NaCl gradient (6,7; see also METHODS, below). The peak corresponding to ^{3}H-E_1 3G was recovered.

Synthesis of E_1 3G-^{14}C

Approximately 50 μCi of UDPGA-^{14}C were incubated with 0.1 μmole of unlabelled E_1 under the conditions described above. DEAE-Sephadex (0 - 0.8M NaCl) chromatography resulted in the clear separation of 2 labelled peaks. The

first eluted was UDPGA-^{14}C or a metabolite thereof, the second corresponded to E_1 3G-^{14}C.

^{3}H-E_1 3G-^{14}C

Appropriate quantities of ^{3}H-E_1 3G and E_1 3G-^{14}C were mixed to yield an approximate ^{3}H/^{14}C ratio of 6 and this was purified by DEAE-Sephadex chromatography, first in 0 - 0.8M NaCl and then in a linear gradient of 0 - 0.4M NaCl, and finally by Celite chromatography in the system iso-octane: t-butanol:M NH_4OH (2:5:5; ref. 12). The purified material had ^{3}H/^{14}C = 5.8. An aliquot of this was completely hydrolyzed by β-glucuronidase (Ketodase). This hydrolysis was 58% inhibited by 5 x 10^{-3} M saccharate (previously boiled in pH 5 buffer) and 81% inhibited by 2 x 10^{-2} M saccharate. The ^{3}H released (rendered ether-soluble) by hydrolysis was shown to be 99% E_1 by TLC in chloroform:ethyl acetate (2:1; ref. 13) with <1% corresponding to E_2. Chromatography on Celite, stationary phase 0.8N NaOH, mobile phase benzene, followed by benzene:ethylene dichloride (1:1; ref. 14) showed a maximum contamination by E_2 of 0.75%. $NaBH_4$ reduction of an aliquot of the synthesized ^{3}H-E_1 3G-^{14}C followed by β-glucuronidase hydrolysis and TLC gave rise to ^{3}H-E_2 (ca. 98%) and ^{3}H-E_1 (<2%) confirming the presence in the original conjugate of a reducible 17-keto group.

Crystallization of the ^{3}H, liberated by β-glucuronidase hydrolysis, with carrier E_1 (calculated SA = 2890 dpm/mg) yielded XLS (crystals) and ML (mother liquor) of SA = 2780 and 2730, respectively. Conversion to the acetate and further crystallization (calc. SA = 2500 dpm/mg) resulted in XLS and ML of SA = 2500 and 2570, respectively.

^{3}H-E_2 3G-^{14}C

An aliquot of the biosynthesized ^{3}H-E_1 3G-^{14}C was reduced with $NaBH_4$ in methanol (7) and then neutralized with acetic acid prior to evaporation, solution in H_2O, and recovery through XAD-2 resin (15). The labelled material was then chromatographed on DEAE-Sephadex (0 - 0.4M NaCl) followed by Celite chromatography as for ^{3}H-E_1 3G-^{14}C (above). The purified material had a ^{3}H/^{14}C ratio of 5.8. An aliquot was completely hydrolyzed by Ketodase and the ^{3}H chromatographed on Celite (16). This showed that the maximum contamination with ^{3}H-E_1 was 0.5%. Crystallization of the ^{3}H released, with carrier E_2 (calc. SA = 2180 dpm/mg), produced

XLS and ML of SA = 2120 and 2300, respectively, while after recrystallization following acetylation (calc. SA = 1670 dpm/mg) these values became 1710 and 1800, respectively.

Injection and urine collection

The labelled conjugates, dissolved in sterile saline, were filtered through Swinnex-13 filters of pore size 0.22 microns (Millipore Corporation, Bedford, Mass.), ethanol was added to a concentration of 10% and 10 ml were injected via an arm vein. The four normal subjects studied were in the first half of the cycle; subjects G and E were injected with ^{3}H-E_1 3G-^{14}C and subjects J and A with ^{3}H-E_2 3G-^{14}C. The amounts of radioactivity administered ranged from 5 to 7 μc of ^{3}H and 0.9 to 1.2 μc of ^{14}C. Urine was collected from subjects G and J at 0 - 6, 6 - 12, 12 - 24, 24 - 48 and 48 - 72 hr. In subjects A and E collections were made at 0 - 3, 3 - 6, 6 - 12, 12 - 24 and 24 - 48 hr. Urine was stored at -15°C until required for analysis.

METHODS

In a preliminary study of the steroidal metabolites in the urine, aliquots of the latter were incubated with bacterial β-glucuronidase and analyzed by Girard separation and Celite partition chromatography (8). The urine remaining following β-glucuronidase hydrolysis and extraction, was subjected to solvolysis (17) in order to split steroid sulfates.

Unhydrolyzed urine was processed on Amberlite XAD-2 resin (15). The resulting conjugate fractions were chromatographed on DEAE-Sephadex in a linear gradient (0 - 0.8M NaCl) essentially as described earlier (7) with some modification. The dimensions of the gel were 0.9 x 58 cm (total bed vol. = 37 ml) and the packed gel occupied a volume of 8 ml/g of dry DEAE-Sephadex. The rate of flow was 45 - 50 ml/hr. The total monoglucosiduronate fractions thus obtained in each case were recovered via XAD-2 resin and further chromatographed on Celite in the iso-octane solvent system described above for the preparation of ^{3}H-E_1 - 3G-^{14}C. Eluted radioactivity was reapplied to DEAE-Sephadex columns as above and chromatographed in the 0 - 0.4 M NaCl linear gradient. Radioactivity was assayed in the various fractions at each stage of these procedures.

Peaks of radioactivity with the chromatographic mobility of E_1 3G were identified as follows. An aliquot was hydrolyzed by Ketodase and the liberated ^3H crystallized with carrier E_1 in the free and acetylated forms until the SA of XLS and ML were constant and equal within the required limits (6). A further aliquot was reduced with $NaBH_4$ (10 mg/ml) in H_2O, prior to pH adjustment to 6 and recovery via XAD-2 resin. This was followed by Celite partition and finally enzymic hydrolysis and crystallization of the ^3H released with carrier E_2 as described above for E_1.

Radioactive peaks corresponding to E_2 3G were identified in suitable aliquots by hydrolysis and crystallization with carrier E_2 as for E_1 above. Further aliquots were methylated with dimethyl sulfate in borate buffer (18) followed by extraction and hydrolysis (6). ^3H-E_2 and ^3H-17β-estradiol-3-methyl ether (E_2 3MeE) were sought by TLC as described elsewhere (6) so as to indicate the presence or absence of a free phenolic group in the original conjugate. On occasion further Celite chromatography of E_2 3G peaks eluted by the 0 - 0.4M NaCl system was performed in order to obtain an additional value for the $^3H/^{14}C$ ratio.

For the peaks recovered from the columns the $^3H/^{14}C$ ratio of each fraction was calculated and the average ratio for each peak obtained. In each case this was checked against the ratio of the pool of the fractions of the peak.

Radioactivity was measured using a Nuclear Chicago Liquid Scintillation Spectrometer as described earlier (19). Quench correction was made by the internal standard technique. All values given are in dpm. For dual labelled samples ^3H and ^{14}C were separately calculated as described by Okita et al (20).

RESULTS

The rates of urinary excretion of ^3H and ^{14}C were similar in the 4 experiments and the mean curve is shown in Fig. 1. The total recoveries of injected ^3H and ^{14}C were 90% and 75%, respectively. Some 70% of each injected isotope was excreted within 3 hr while from 3 to 6 hr a further 8%

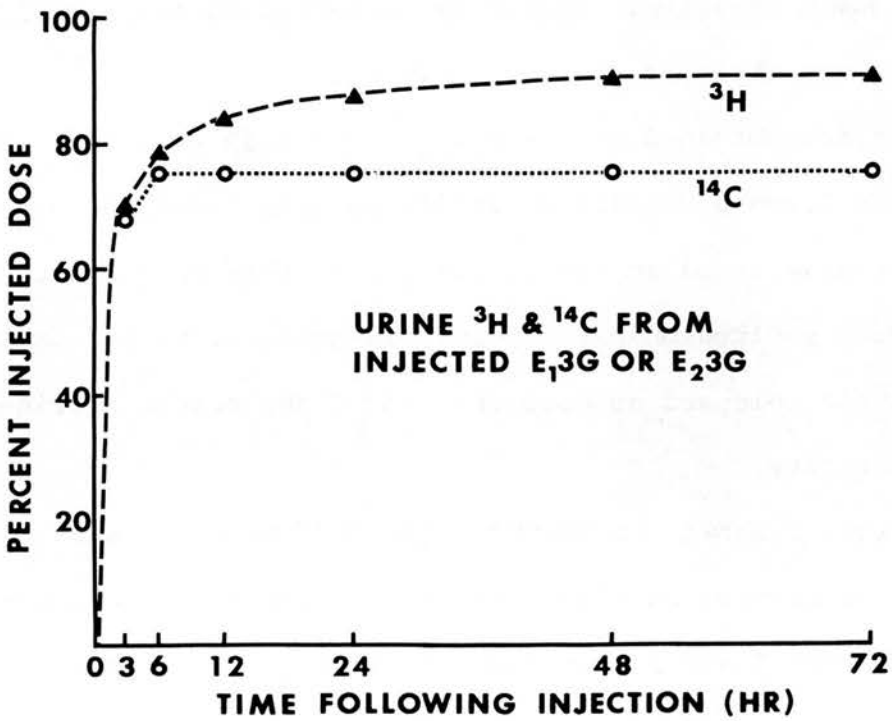

Fig. 1. Cumulative excretion of ^{3}H and ^{14}C following injection of ^{3}H-E_1 3G-^{14}C or ^{3}H-E_2 3G-^{14}C into normal females.

of the ^{3}H dose was excreted along with about 5% of the ^{14}C dose. Beyond 6 hr no further ^{14}C could be detected whereas an additional 12% of the ^{3}H dose appeared from 6 to 48 hr after administration. Little or no excretion of either isotope was observed from 48 to 72 hr.

Urines obtained at 0 - 6, 6 - 12 and 12 - 24 hr from subjects G and J contained, following hydrolysis, no identifiable labelled metabolites other than E_1 and E_2. In each case β-glucuronidase hydrolysis appeared to be complete. Solvolysis released no more than 1% of the original urine radioactivity.

Table I shows the $^{3}H/^{14}C$ ratios of the conjugate fractions at various stages of the purification procedure for subjects G and J. In each urine aliquot two peaks, corresponding to E_1 3G and E_2 3G were found after elution of the DEAE-Sephadex column with 0 - 0.4M NaCl. For the 0 - 6 hr fractions ^{3}H and ^{14}C were found in each peak. The patterns shown in Figs. 2 and 3 for G (0 - 6; E_1 3G injected) and J (0 - 6; E_2 3G injected) are typical of the separations achieved in this study. It appeared from these that the transformations E_1 3G $\rightarrow$ E_2 3G and E_2 3G $\rightarrow$ E_1 3G could both occur, particularly the latter, and, in view of the $^{3}H/^{14}C$ ratios of the metabolites it was clear that these conversions could involve the intact conjugates. Beyond 6 hr

TABLE I

$^{3}H/^{14}C$ Ratios of metabolites at various stages of purification for subjects G and J*

Purification step	Conjugate fractions G		J	
	0 - 6	6 - 24	0 - 6	6 - 24
Urine through XAD-2	6.8	**	6.7	**
0 - 0.8M NaCl	6.1	**	5.8	**
Celite partition	6.0	76	6.1	**
0 - 0.4M NaCl (peak 1-E_1 3G)	6.2	62	6.0	**
" " (" 2-E_2 3G)	7.5	**	6.1	**
Peak 1, reduced → Celite	6.1	†	6.2	†
Peak 2 → Celite	7.5	†	6.0	†

*^{3}H-E_1 3G-^{14}C injected into G; ^{3}H-E_2 3G-^{14}C into J; $^{3}H/^{14}C$ = 5.8 for both.
**Apparently infinity.
†Not done.

little or no ^{14}C was associated with the urinary ^{3}H-labelled conjugates (Table I). The results in Table II for subjects A and E are in agreement with those of Table I. The $^{3}H/^{14}C$ ratios of the various metabolites in these experiments were relatively constant throughout the purification procedure employed. The total monoglucosiduronate fractions were separated from most of the interfering urinary chromogens by the 0 - 0.8M NaCl gradient although significant amounts of chromogens were still present. Celite partition chromatography removed most of the latter while in the 0 - 0.4M NaCl gradient the glucosiduronate peaks were purified to a degree where their solutions were colourless.

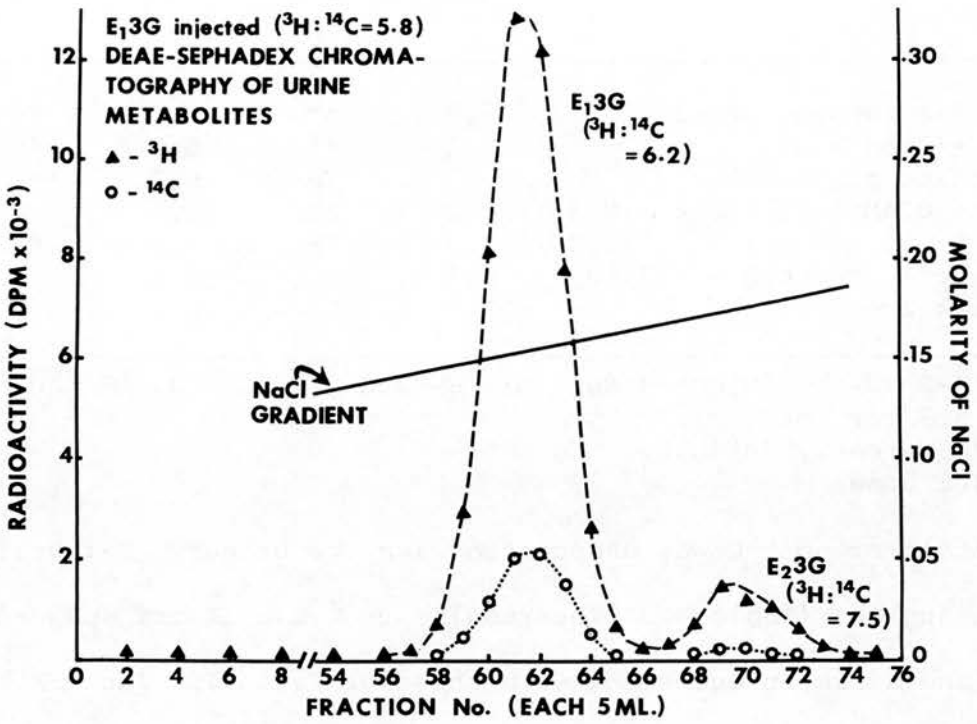

Fig. 2. DEAE-Sephadex chromatography (0 - 0.4M NaCl) of urinary metabolites of ^{3}H-E_1 3G-^{14}C in subject G from 0 to 6 hr after injection.

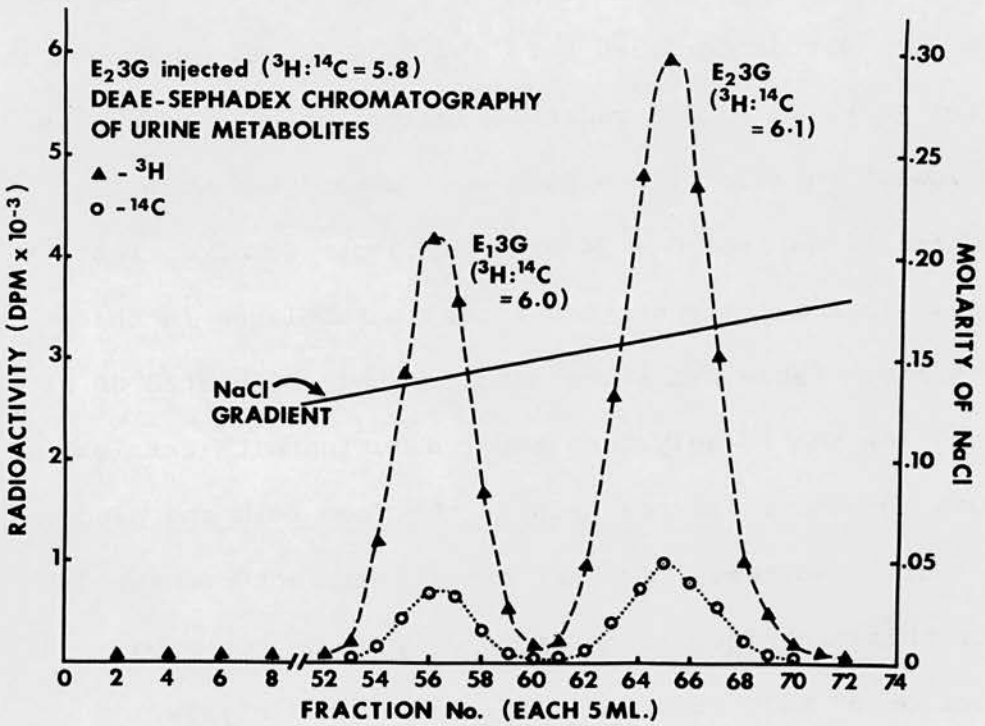

Fig. 3. DEAE-Sephadex chromatography (0 - 0.4M NaCl) of urinary metabolites of ^{3}H-E_2 3G-^{14}C in subject J from 0 to 6 hr after injection.

TABLE II

$^{3}H/^{14}C$ Ratios of metabolites at various stages of purification for subjects A and E*

Purification step	Conjugate fractions			
	A		E	
	0 - 3	3 - 6	0 - 3	3 - 6
Urine through XAD-2	6.0	9.4	6.1	8.7
0 - 0.8M NaCl	5.9	10.5	5.7	8.4
Celite partition	6.1	10.7	5.8	8.9
0 - 0.4M NaCl (peak 1-E_1 3G)	5.9	13.5	5.7	8.1
" " " (" 2-E_2 3G)	5.8	7.1	6.0	9.2

*^{3}H-E_1 3G-^{14}C injected into E; ^{3}H-E_2 3G-^{14}C into A; $^{3}H/^{14}C$ = 5.8 for both.

Reverse isotope dilution of the ^{3}H-aglycones derived from the various purified E_1 3G and E_2 3G fractions showed the latter to be 90 - 100% radiochemically pure in most cases. The lowest purities (79 - 86%) were associated with the E_2 3G fractions from 6 - 24 hr in subjects G and J, respectively. This may suggest additional metabolites in these fractions. Table III shows data for the calculated SA values for the ^{3}H-aglycones after dilution with carrier, SA values for the final crystals in the free form and also in the acetylated form. Further details are not presented since this technique is now so widely used. Chemical reduction of E_1 3G fractions followed by hydrolysis and crystallization of the ^{3}H-aglycone with carrier E_2 confirmed the presence of a free 17-keto group in the original conjugate. Methylation of E_2 3G fractions followed by hydrolysis resulted in identification of at least 95% of the radioactivity as being associated with free E_2, thus strongly indicating the absence of a free phenolic group in the original conjugate. These data, together with the behaviour on DEAE-Sephadex and Celite partition columns, established the metabolites as the 3-glucosiduronates of E_1 and E_2. In no instance was any evidence obtained for the presence of labelled E_2 17G.

Table IV summarizes the amounts (in terms of ^{3}H) and the final $^{3}H/^{14}C$ ratios of the metabolites isolated from 0

TABLE III

Crystallization of ^{3}H-steroids derived from the E_1 3G and E_2 3G fractions

Experiment	Metabolite fraction	SA (dpm/mg) Calculated*	XLS (free)	XLS (acetates)†
G (E_1 3G injected)	E_1 3G(0-6)	6,340	6,430	6,370
	E_2 3G(0-6)	2,860	2,790	2,840
	E_1 3G(6-24)	6,720	6,840	6,780
	E_2 3G(6-24)	1,920	1,470	1,510
J (E_2 3G injected)	E_1 3G(0-6)	3,300	3,250	3,100
	E_2 3G(0-6)	6,150	5,670	5,890
	E_1 3G(6-24)	7,080	7,040	7,150
	E_2 3G(6-24)	1,550	1,270	1,330
E (E_1 3G injected)	E_1 3G(0-3)	12,900	12,670	12,500
	E_2 3G(0-3)	10,000	9,150	9,140
	E_1 3G(3-6)	8,300	8,070	7,810
	E_2 3G(3-6)	1,700	1,550	1,520
A (E_2 3G injected)	E_1 3G(0-3)	2,620	2,670	2,560
	E_2 3G(0-3)	4,810	4,360	4,340
	E_1 3G(3-6)	2,750	2,550	2,540
	E_2 3G(3-6)	2,080	1,870	1,880

*Following dilution of a known no. of dpm ^{3}H with a known wt. of carrier.
†Values corrected to refer to free steroid.

to 6 hr and from 6 - 24 hr in subjects G and J. After E_1 3G injection in subject G, 79% of the total urinary ^3H identified in the 0 - 24 hr urine was excreted in the first 6 hr as E_1 3G with a $^3H/^{14}C$ ratio (6.1) slightly higher than that injected. A further 9% appeared within 6 hr as E_2 3G of isotope ratio 7.5 indicating that it arose mainly by direct reduction of E_1 3G. Beyond 6 hr, although very small amounts of metabolites were excreted, E_1 3G predominated over E_2 3G. The absence of ^{14}C suggested deconjugation followed by equilibrium between E_1 and E_2 prior to reconjugation with unlabelled UDPGA and finally urinary excretion. Injection of E_2 3G (subject J, Table IV) was followed by a very considerable direct conversion to E_1 3G, 29% of the ^3H present in the 0 - 24 hr urine being excreted in 6 hr as the latter compound, with $^3H/^{14}C$ = 6.1. As in the case of injected E_1 3G no ^{14}C was associated with the metabolites in the period 6 - 24 hr during which time ^3H-E_1 3G was approximately four times greater than ^3H-E_2 3G.

Table V contains data for the metabolism of injected E_1 3G and E_2 3G in subjects E and A, respectively, over the periods 0 - 3 and 3 - 6 hr. Excellent evidence was obtained for a direct E_2 3G → E_1 3G conversion, 32% of the ^3H identified in 0 - 6 hr after E_2 3G injection appearing in the first 3 hr in the form of E_1 3G with $^3H/^{14}C$ = 5.9. In this same experiment it was also evident that some deconjugation

TABLE IV

Relative amounts and $^{3}H/^{14}C$ ratios of metabolites in subjects G and J

Expt	Urine metab	^{3}H (0 - 6) dpm* $\times 10^{-6}$	% of † 0-24 hr	$^{3}H/^{14}C$	^{3}H (6 - 24) dpm* $\times 10^{-6}$	% of † 0-24 hr	$^{3}H/^{14}C$
G (E_1 3G injected)	E_1 3G	5.17	79	6.1	0.594	9	62
	E_2 3G	0.615	9	7.5	0.16	3	‡
J (E_2 3G injected)	E_1 3G	1.43	29	6.1	0.602	12	‡
	E_2 3G	2.76	56	6.1	0.173	3	‡

*Uncorrected for procedural losses during extraction and purification.
†% of total ^{3}H identified as E_1 3G + E_2 3G in 0 - 24 hr.
‡Apparently infinity.

TABLE V

Relative amounts and $^{3}H/^{14}C$ ratios of metabolites in subjects A and E

Expt	Urine metab	^{3}H (0 - 3) dpm* $\times 10^{-6}$	% of † 0-6 hr	$^{3}H/^{14}C$	^{3}H (3 - 6) dpm* $\times 10^{-6}$	% of † 0-6 hr	$^{3}H/^{14}C$
A (E_2 3G injected)	E_1 3G	1.74	32	5.9	0.243	5	13.5
	E_2 3G	3.25	61	5.8	0.118	2	7.1
E (E_1 3G injected)	E_1 3G	6.06	88	5.7	0.360	5	8.1
	E_2 3G	0.448	6	6.0	0.06	1	9.2

*Uncorrected for procedural losses during extraction and purification.
†% of total ^{3}H identified as E_1 3G + E_2 3G in 0 - 6 hr.

had occurred as reflected in the $^{3}H/^{14}C$ ratios of the 3 - 6 hr metabolites (E_1 3G = 13.5, E_2 3G = 7.1). This appeared to be associated with an increase in the ratio of E_1 3G/E_2 3G at this time as compared with the 0 - 3 hr period. Table V also shows a small, though definite, conversion of E_1 3G to E_2 3G within 3 hr and an increase in the $^{3}H/^{14}C$ ratios of the urinary metabolites of E_1 3G at 3 - 6 hr (E_1 3G = 8.1, E_2 3G = 9.2).

In the two experiments where E_1 3G was injected the patterns of urinary metabolites during 0 - 6 hr (ca 80% of the ^{3}H dose excreted) were such that the E_1 3G:E_2 3G ratios with respect to ^{3}H were 13:1 and 9:1. In the experiments in which E_2 3G was injected these ratios were 0.61:1 and 0.59:

DISCUSSION

The above results clearly indicate the interconversion of E_1 3G and E_2 3G in the human in vivo, without prior removal of the glucuronic acid moiety. The transformation is markedly in favour of E_1 3G production and occurs within 3 hr of intravenous injection of the conjugates. Beyond 3 hr there is evidence for some deconjugation although this is not a major process in the metabolism of E_1 3G or E_2 3G. Such deconjugation could, conceivably, be occurring in the gut, a site already shown to be active in this respect where the glucosiduronates of estriol are concerned (21, 22). No

labelled phenolic aglycones other than E_1 or E_2 could be detected in the present work nor was there any reason to suspect the presence of E_2 17G in any of the urines analyzed. This, however, does not exclude the possible presence of minor amounts of other compounds, particularly between 24 and 48 hr.

Zucconi et al (3), following introduction of ^{3}H-E_1 3G-^{14}C into the human fetus in situ, recovered about 1 - 2% of the radioactivity in the maternal urine and identified this largely as E_1 3G of $^{3}H/^{14}C$ ratio equal to that administered together with very small amounts of labelled E_2 following β-glucuronidase hydrolysis. In view of the small degree of conversion of E_1 3G to E_2 3G in the present work it seems possible that a similarly small fraction of the E_1 3G transferred to the mother could have been converted to E_2 3G, thus accounting for the findings of Zucconi et al.

It appears obvious that the metabolism of the 3-glucosiduronates of E_1 and E_2 is very different from that of E_2 17G. Thus the major part of intravenously injected E_2 17G has been shown to be metabolized to E_1 3G and E_2 3G with the former compound predominating (6). This, of necessity, would involve considerable removal of the 17-glucosiduronate group. Therefore it would appear that glucuronic acid esterified at C-3 of E_1 or E_2 is much less liable to be re-

moved following intravenous injection in the human than is the case when it is esterified at C-17 of E_2.

It was speculated earlier (6) that E_2 17G might be metabolized via a diglucosiduronate form to E_2 3G and hence to E_1 3G. In order for this to be so it was necessary to show that E_2 3G could be directly dehydrogenated to E_1 3G. The results in the present paper now show such a conversion to be a distinct possibility.

ACKNOWLEDGMENT

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4. The following trivial names and abbreviations for steroids and their conjugates are employed in this paper; estrone (E_1) = 3-hydroxyestra-1,3,5(10)-trien-17-one; 17β-estradiol (E_2) = estra-1,3,5(10)-triene-3,17β-diol; estriol = estra-1,3,5(10)-triene-3,16α,17β-triol; estrone-3-glucosiduronate (E_1 3G) = 17-oxoestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-3-glucosiduronate (E_2 3G) = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-17-glucosiduronate (E_2 17G) = 3-hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate.

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MÉTABOLISME DES GLUCURONATES * D'OESTROGÈNE ADMINISTRÉS PAR VOIE ORALE À LA FEMME NORMALE

R. HOBKIRK, M. NILSEN et P. MUSEY [1]

INTRODUCTION

Des études récentes démontrent clairement que, chez la femme normale, le métabolisme du 17β-œstradiol-17 glucuronate * ($E_2$17G) circulant diffère considérablement de celui du 17β-œstradiol-3-glucuronate ($E_2$3G). [1, 2] Ainsi, le $E_2$17G est fortement déconjugué (à environ 65%) en œstrone-3-glucuronate ($E_1$3G) et $E_2$3G comme métabolites urinaires principaux, [1,3] alors qu'environ 30% sont éliminés dans les urines sous forme de $E_2$17G [1,4] (probablement la substance injectée intacte). L'administration intraveineuse de $E_2$3G (ou de $E_1$3G) marqués, en revanche, est suivie d'une élimination rapide de la radioactivité dans les urines (70% en 3 heures) sans qu'il se soit produit de déconjugaison. [2] Après trois heures, on constate une déconjugaison légère (10-15%). [2] Il semble probable que la déconjugaison, surtout celle des glucuronates d'œstriol, se produit dans le tractus intestinal. [5,6] Si tel est le cas pour les conjugués de l'œstrone (E_1) et du 17β-œstradial (E_2), la différence notée ci-dessus pour le métabolisme du $E_2$17G et du $E_2$3G pourrait s'expliquer par un degré différent de participation des deux composés dans le système entéro-hépatique. Pour tenter de mieux comprendre le métabolisme de ces composés, nous avons étudié le destin de l'$E_1$3G et de l'$E_2$17G ingérés par la femme. Nous présentons ici cette étude, et en comparons les résultats avec d'autres données provenant de notre laboratoire.

MATÉRIEL ET MÉTHODES

L'étude a été effectuée au cours de la première moitié du cycle menstruel de sujets volontaires et en bonne santé. On leur a administré des œstrogènes libres ou conjugués par voie intraveineuse (une seule injection) en solution d'éthanol-saline 10% (v/v), [7] les conjugués étant administrés par la bouche dans l'H_2O et les œstrogènes libres dans l'éthanol-H_2O à 10% (v/v). Les doses de ^3H administrées sous forme conjuguée étaient de 5μc pour le sujet LO, 10μc pour VA, et 25μc pour CL. Les urines ont été recueillies à intervalles appropriés et congelées jusqu'au moment de l'analyse. Tous les réactifs chimiques, solvants organiques, enzymes, substances nécessaires à la chromatographie, etc. ont été obtenus et le cas échéant purifiés dans les conditions décrites ailleurs. [7] Nous avons acheté le 17β-œstradiol-6,7-^3H et le 17-β-œstradiol-6,7-^3H-17-glucuronate à la New England Nuclear Corp. (Boston, Massachusetts) et les avons purifiés comme nous l'avons exposé ailleurs. [7,8] L'œstrone-6, 7- ^3H-3-glucunorate-^{14}C (marqué de façon non spécifique) a été préparé par biosynthèse et purifié ainsi que nous l'avons déjà exposé. [2] Toutes les mesures de radioactivité ont été réalisées à l'aide de spectromètres à scintillation liquide « Nuclear Chicago » ainsi que nous en avons fait état ailleurs. [7] L'analyse des conjugués de l'urine a été faite par chromatographie séquentielle sur résine Amberlite XAD-2 [9], Sephadex DEAE avec des gradients de concentration de NaCl [2,8] et des colonnes de fractionnement en Celite. [10] Après avoir séparé les conjugués, nous avons identifié les stéroïdes par hydrolyse avec la *β-glucuronidase* suivie d'un fractionnement de Girard et d'une chromatographie sur colonne de fractionnement en Celite. [11] Ces opérations ont été suivies de la cristallisation à activité spécifique constante à l'aide de stéroïdes porteurs avant et après formation d'acétate. [1,2,7]

RÉSULTATS

La Fig. 1 montre l'excrétion de la radioactivité après administration buccale de $E_1$3G ou de $E_2$3G, $E_2$17G et E_2 marqués. On constate un retard d'au moins trois heures pour l'excrétion après l'administration des conjugués, mais non après celle du stéroïde libre. Au delà de 3-6 heures, la courbe d'excrétion dans le cas des conjugués est semblable à celle d'au delà de 6 heures après l'injection intraveineuse de $E_2$17G (Fig. 2). L'analyse des conjugués urinaires après l'administration buccale de $E_1$3G marqué à $^3H/^{14}C$ dans deux expériences produit les données qui figurent dans les Tableaux I et II. En dehors de la période de 0-3 heures, où 1-2% de la dose administrée a été éliminée dans un rapport défini $^3H/^{14}C$, il n'a pas été retrouvé de ^{14}C dans l'urine,

* L'expression « glucuronate » est ci-après utilisée comme synonyme de « glucosiduronate », terme employé dans le texte anglais.

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alors qu'environ 83% de la dose de ^{3}H a été éliminé en 96 heures. L'$E_2$17G n'a pas été identifié comme métabolite urinaire (Fig. 3) mais $E_1$3G et $E_2$3G représentaient à eux deux au moins 90% de la radioactivité de l'urine pour la période de 0-48 heures lorsqu'une analyse détaillée a été faite. Il ressort

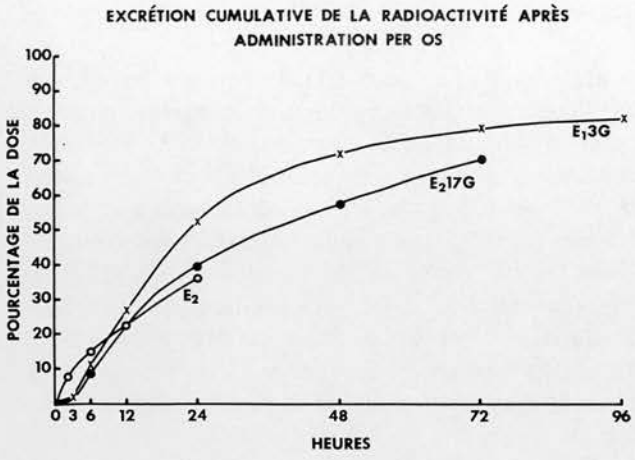

Fig. 1 — Excrétion urinaire cumulative de la radioactivité (^{3}H) après administration per os de $^{3}H/^{14}C$-$E_1$3G, ^{3}H-$E_2$17G et ^{3}H-E_2.

aussi des Tableaux I et II que le(s) monoglucuronate(s) d'œstriol (E_3) ne représentai(en)t pas plus de 3-5% de la radioactivité enregistrée dans l'urine pendant les deux premiers jours. La contribution de E_3 a augmenté pendant cette période et il est fort possible qu'elle se serait accrue pendant les deux jours suivants qui n'ont pas été étudiés en détail. Nous avons pu exclure la possibilité que des quantités significatives d'autres métabolites d'œstrogène soient présentes pendant la période de 0-48 heures.

Le Tableau III montre les métabolites urinaires identifiés après administration buccale de $E_2$17G. Le schéma ressemble beaucoup à celui décrit ci-des-

TABLEAU I

Métabolites urinaires ^{3}H (en pourcentage de ^{3}H identifié) après administration per os de $^{3}H/^{14}C$-$E_1$3G ($^{3}H/^{14}C$ = 5.8) au sujet LO.

Heures après administration	*$E_1$3G*	*$E_2$3G*	*E_3G **
0 — 3	1.2 **	0	0
3 — 6	16.0	0.5	0
6 — 12	17.5	2.3	0.4
12 — 24	30.5	5.7	1.1
24 — 48	18.3	3.0	3.6
Total	83.5	11.5	5.1

* Position de conjugaison avec l'acide glucuronique non déterminée.

** Accompagné de ^{14}C ($^{3}H/^{14}C$ = 5.8). Il n'a pas été trouvé d'autre ^{14}C.

TABLEAU II

Métabolites urinaires au ^{3}H (en pourcentage de ^{3}H identifié) après administration per os de $^{3}H/^{14}C$-$E_1$3G ($^{3}H/^{14}C$ = 5.8) au sujet VA.

Heures après administration	*$E_1$3G*	*$E_2$3G*	*E_3G **
0 — 3	3.9 **	0.3	< 0.01
3 — 6	10.6	1.6	0.05
6 — 12	25.0	6.0	0.3
12 — 24	21.6	6.9	0.8
24 — 48	16.5	4.3	2.2
Total	77.6	19.1	3.3

* Position de conjugaison avec l'acide glucuronique non déterminée.

** Accompagné de ^{14}C ($^{3}H/^{14}C$ = 10.6). Il n'a pas été trouvé d'autre ^{14}C.

sus pour l'$E_1$3G per os. Il n'a pas été trouvé de $E_2$17G dans l'urine et les $E_1$3G + $E_2$3G représentaient pour ainsi dire la totalité de la radioactivité éliminée en 48 heures. Une très faible quantité de E_3 conjugué a été décelée; elle semblait s'accroître au cours de la période de 48 à 72 heures (ne figure pas dans le Tableau).

DISCUSSION

Fishman et coll [12] ont signalé qu'une fraction notable d'une dose de E_2 administrée par voie buccale est rapidement absorbée et éliminée dans l'urine sous forme de conjugués glucuronates de E_2 et E_1, c'est-à-dire qu'il ne se produit plus guère de conversion ultérieure de la portion stéroïde. Toutefois, environ 60% de la dose suivait un métabolisme similaire à celui de l'E_2 administré par injection intraveineuse avec la production de quantités abondantes de métabolites 2-hydroxy et 16-hydroxy. Il ressort clairement, de la présente étude que, d'après l'analyse des métabolites urinaires, tant l'$E_1$3G que l'$E_2$17G sont considérablement sinon complètement déconjugués dans le cours du métabolisme à la suite de l'administration buccale. Sans pouvoir écarter la possibilité que l'es-

TABLEAU III

Métabolites urinaires marqués au ^{3}H (en pourcentage de ^{3}H identifié) après administration per os de ^{3}H-$E_2$17G au sujet CL.

Heures après administration	*$E_1$3G*	*$E_2$3G*	*E_3G **
0 — 3	1.2	0.6	< 0.1
3 — 6	9.9	4.9	< 0.1
6 — 12	18.3	7.5	< 0.1
12 — 24	22.7	6.4	0.3
24 — 48	20.0	5.5	2.8
Total	72.1	24.9	3.1

* Position de conjugaison avec l'acide glucuronique non déterminée.

tomac puisse être un site de déconjugaison dans nos expériences, il semble improbable qu'une hydrolyse importante puisse s'y produire, parce que le stéroïde libre ainsi produit aurait vraisemblablement été métabolisé de la façon indiquée par Fishman et ses collaborateurs pour l'E_2 libre. Or le contraire ressort clairement de notre constatation, à savoir que l'administration buccale de $^3H/^{14}C$-$E_1$3G et ^3H-$E_2$17G ne produit guère autre chose que ^3H-$E_1$3G et ^3H-$E_2$3G dans l'urine. En outre, le retard d'excrétion des métabolites après l'administration buccale des conjugués (1-2% de la dose en trois heures) diffère de l'excrétion constatée après l'absorption de E_2 libre, où 7,5% de la dose ont été éliminés dans l'urine dans les deux heures (Fig. 1). Quel que soit le site exact où intervient la déconjugaison (hydrolyse) des glucuronates dans le système gastro-intestinal, il est tentant de considérer que cette étape pourrait être le facteur limitant de réabsorption, puisque l'E_2 (libre) administré buccalement est rapidement absorbé[12] et que les 3-glucuronates, une fois dans la circulation sanguine, sont très rapidement excrétés dans l'urine[2] (Fig. 2).

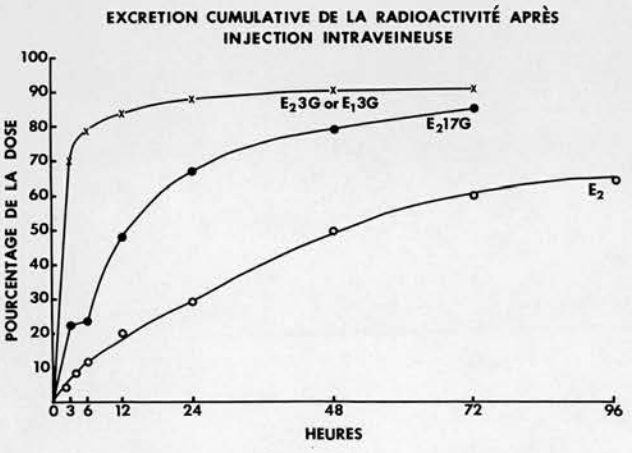

Fig. 2 — Excrétion urinaire cumulative de la radioactivité (^3H) après injection intraveineuse de $^3H/^{14}C$-$E_2$3G ou $E_1$3G, ^3H-$E_2$17G et ^3H-E_2.

Le manque relatif de métabolites stéroïdaux autres que $E_1$3G et $E_2$3G dans l'urine, à la suite de l'administration buccale de $E_1$3G ou de $E_2$17G, donne plutôt à penser que les stéroïdes ont pénétré dans le système porte sous forme conjuguée plutôt que sous la forme libre, autrement dit que le tractus gastro-intestinal est un site de déconjugaisons *et* de reconjugaison.

Il est intéressant de s'interroger sur la façon dont il faut interpréter la courbe d'excrétion de la radioactivité à la suite d'une injection intraveineuse de ^3H-$E_2$17G (Fig. 2). Nous avons déjà établi que l'excrétion initiale rapide (0-3 heures) est due presque entièrement à ^3H-$E_2$17G joint à une très faible quantité de ^3H-17β-œstradiol-3-sulfate-17-glucuronate ($E_2$3S17G).[4] Le retard d'excrétion, qui se produit entre environ 3 et 6 heures, est similaire à celui que l'on constate après les 3 premières heures à peu près qui suivent l'administration buccale de $E_2$17G (Fig. 1). L'excrétion de ^3H au delà de 6 heures dans l'expérience avec injection intraveineuse est très semblable à celle que l'on note après l'administration

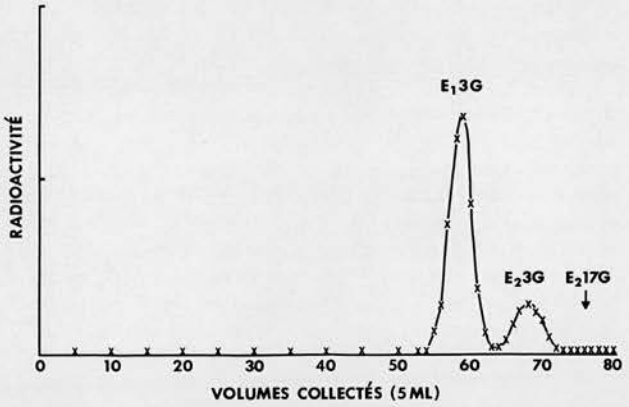

Fig. 3 — Fractionnement typique sur colonne Sephadex DEAE de conjugués ^3H dans les urines après administration orale de $^3H/^{14}C$-$E_1$3G au sujet VA. Ce diagramme concerne les conjugués urinaires entre 12 et 24 heures après administration du composé marqué.

per os de $E_2$17G: presque toute la radioactivité urinaire se retrouve sous forme de ^3H-$E_1$3G et ^3H-$E_2$3G[4]. Cela pourrait signifier qu'une partie de l'$E_2$17G injecté est excrétée dans la bile (peut-être sous forme de $E_2$3S17G) avec déconjugaison ultérieure et réabsorption, et enfin excrétion urinaire des 3-glucuronates des E_1 et E_2 reconjugués.

Résumé

De l'œstrone-6,7-^3H-3-glucuronate-^{14}C (marqué de façon non spécifique) a été administré buccalement à deux jeunes femmes normales. Pendant les 3 premières heures, 1-2% seulement de la dose a été excrété dans les urines, cette excrétion contenant à la fois ^3H et ^{14}C. Par la suite, 83% de la dose de ^3H est apparu dans les urines en quatre jours, mais il n'a plus été retrouvé de ^{14}C. Au moins 90% du ^3H des 48 heures représentait de l'œstrone-3-glucuronate et du 17β-œstradiol-3-glucuronate et 3-5% correspondait à un (ou des) glucuronate(s) d'œstriol. De même a-t-on noté lors de l'administration de 17β-œstradiol-6,7-^3H-17-glucuronate, un retard d'environ 3 heures dans l'excrétion, et l'on retrouvait 85% de la dose radioactive dans les urines en 3 jours. Le ^3H excrété en 48 heures était presque entièrement sous la forme d'œstrone-3-glucuronate et de 17β-œstradiol-3-glucuronate. Ces données démontrent l'efficacité du tractus gastro-intestinal à déconjuguer des glucu-

ronates d'œstrogène et peut-être aussi à les reconjuguer avant l'excrétion urinaire.

Summary

Estrone-6, 7-^{3}H-3-glucosiduronate-^{14}C (generally labelled) was administered orally to two normal young human females. Over the first 3 hr. only 1-2% of the dose was excreted in the urine and this contained both ^{3}H and ^{14}C. Beyond this time, some 83% of the ^{3}H dose appeared in the urine over a period of 4 days, but no further ^{14}C was recovered. At least, 90% of the ^{3}H in 48 hr. consisted of estrone-3-glucosiduronate and 17β-estradiol-3-glucosiduronate while 3-5% corresponded to estriol glucosiduronate (s). When 17β-estradiol-6, 7-^{3}H-17-glucosiduronate was similarly administered, a lag in excretion of about 3 hrs. was again seen, followed by recovery in the urine of 85% of the radioactive dose within 3 days. The ^{3}H excreted in 48 hrs. was almost completely in the form of estrone-3-glucosiduronate and 17β-estradiol-3-glucosiduronate. These data indicated the efficiency of the gastrointestinal tract in deconjugating the estrogen glucosiduronates and perhaps also in reconjugation prior to urinary excretion.

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Nous remercions le docteur P.R. Blahey de sa collaboration clinique à notre étude. Ces travaux ont été supportés grâce à des fonds (MT532) octroyés par le Conseil de la Recherche Médicale du Canada. R.H. est associé de recherche du Conseil de la Recherche Médicale et P.M. reçoit une subvention de la Canadian International Development Agency. P.M. prépare un doctorat en philosophie à la section Biochimie de l'Université McGill.

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Metabolism of 17β-Estradiol to 17β-Estradiol-3-glucosiduronate and 17β-Estradiol-17-glucosiduronate by the Normal Human Female

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ABSTRACT. 6,7-^{3}H-17β-estradiol (estradiol) was administered intravenously into 2 normal females and orally to 2 others. Urines collected over various time intervals were analyzed for ^{3}H-estradiol-3-glucosiduronate and ^{3}H-estradiol-17-glucosiduronate by Amberlite resin, DEAE-Sephadex and Celite partition chromatography followed by enzyme hydrolysis, derivative formation and reverse isotope dilution. In the first 2 hr after intravenous injection urinary estradiol-17-glucosiduronate was more than 4 times greater in amount than the 3-monoglucosiduronate. Of the total estradiol-17-glucosiduronate excreted in the first 24 hr after injection, at least 96% was in the first 2 hr, whereas estradiol-3-glucosiduronate showed a gradual increase throughout the 24-hr period. After ingestion of ^{3}H-estradiol, no firm evidence was obtained for the presence of urinary estradiol-17-glucosiduronate, whereas estradiol-3-glucosiduronate appeared to be excreted at a greater rate than following iv injection. It is concluded that both monoglucosiduronates of estradiol are urinary metabolites of intravenous estradiol but only the 3-glucosiduronate is a urinary metabolite of the ingested hormone. (*J Clin Endocr* **32:** 779, 1971)

LITTLE appears to be known regarding the naturally occurring glucosiduronate form(s) of 17β-estradiol (hereafter referred to as estradiol or E_2)[2] in the human subject. Data obtained elsewhere suggest the presence of estradiol-17-glucosiduronate ($E_2$17G) in pregnancy urine (1, 2) and one of these reports further suggests that the 17- and 3-monoglucosiduronates of E_2 may be present in approximately equal amounts (2). More recently, the presence of labeled $E_2$17G in urine following injection of ^{14}C-E_2 to humans has been indicated on the basis of chromatographic mobility (3). The present communication deals with the identification of both monoglucosiduronates of E_2 as urinary metabolites of administered ^{3}H-E_2 in the human female and with the sequence in which they arise.

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[2] The following trivial names and abbreviations are used; estradiol-17-glucosiduronate (E_2 17G) = 3-hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate; estradiol-3-glucosiduronate ($E_2$3G) = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; estradiol-3-sulfate-17-glucosiduronate ($E_2$3S17G) = 3-sulfo-estra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate; $E_2$3MeE = estradiol-3-methyl ether.

Materials and Methods

Labeled steroids and conjugates. 6,7-^{3}H-E_2 of specific activity (SA) = 39 Ci/mmole was purchased from New England Nuclear Corp. (NENC), Boston, Mass., and was at least 98% pure as judged by crystallization with carrier following column partition chromatography (CPC) on Celite (4).

4-^{14}C-estradiol-3-glucosiduronate ($E_2$3G) was biosynthesized as described elsewhere (5) by incubating ^{14}C-E_2 (NENC, SA = 52 mCi/mmole) with unlabeled uridine diphosphate glucuronic acid and a guinea pig liver homogenate. The mixture of ^{14}C-$E_2$3G and ^{14}C-estrone-3-glucosiduronate ($E_1$3G) so formed was reduced with $NaBH_4$ (5) and the resulting ^{14}C-$E_2$3G was purified by DEAE-Sephadex and Celite CPC (5). Hydrolysis followed by crystallization with carrier E_2 showed that some 98% of the ^{14}C-aglycone was in the form of the latter steroid. Hydrolysis was efficiently inhibited by saccharo-1,4-lactone.

4-^{14}C-$E_2$17G of SA = 45.2 mCi/mmole was synthesized by NENC. Following chromatography on DEAE-Sephadex in a linear gradient (0–0.4M) of NaCl (6), a portion was mixed with purified 6,7-^{3}H-$E_2$17G (NENC, SA = 1.0 Ci/mmole) to yield a ^{3}H/^{14}C ratio = 7.5. This was methylated (7) and hydrolyzed (7) to give an ether-soluble fraction of isotope ratio = 7.3. Crystallization with unlabeled estradiol-3-

methyl ether ($E_2$3MeE) resulted in crystals and mother liquor of ratios 7.3 and 7.2, respectively.

Reagents, chemicals, etc. All organic solvents, chemicals, Celite, etc., were of analytical grade and were purified where necessary by published procedures (4, 8). All unlabeled steroids were purchased from Mann Research Laboratories, Inc., New York, and were checked for purity by melting point determination (microscope hot stage) and thin-layer chromatography. DEAE-Sephadex (A-25) was a product of Pharmacia (Canada) Ltd., Montreal. Beef liver β-glucuronidase (Ketodase) was purchased from Warner-Chilcott, Morris Plains, N. J. Saccharo-1,4-lactone was purchased from Calbiochem, Los Angeles, Cal.

Administration of ^3H-E_2. This was injected, as described elsewhere (4), in a single dose via an arm vein into 2 normal female volunteers; subject CR (received 25.6 μCi) and subject DE (received 18 μCi). It was also administered orally in 10% (v/v) ethanol-H_2O to 2 further normal females; subject JN (received 25.5 μCi) and subject JS (received 22.4 μCi). The 4 subjects were aged 21 to 25 yr and were at about day 10 of the menstrual cycle. Urine was collected at 2 hr intervals up to 6 hr and again at 12 and 24 hr. In subject JN the 4 and 6 hr collections were inadvertently mixed, leading to a 2–6 hr collection. All urine was frozen until analyzed.

Analysis of urinary conjugates. Suitable urine volumes (corresponding to no less than 1 hr and no greater than 6 hr collections) were processed on Amberlite XAD-2 resin (9) followed by DEAE-Sephadex chromatography (58 cm column, 0–0.8M NaCl gradient, 10 ml fractions collected; ref. 6). The "total" monoglucosiduronate fraction so obtained (fractions 20–40 pooled) was chromatographed on Celite in isooctane:*t*-butanol:1M NH_4OH (2:5:5; ref. 10) and then on a 0.9×95 cm DEAE-Sephadex column in a linear gradient (500 ml H_2O in mixing vessel, 500 ml 0.8M NaCl in donor vessel) collecting 5 ml fractions. Purified ^{14}C-$E_2$3G or ^{14}C-$E_2$17G or both were added at various points of the procedure to act as internal standards for purposes of checking recovery and as chromatographic markers. Peaks containing $E_2$3G were incubated with β-glucuronidase (500 U/ml for 20 hr at 38 C) with and without inhibitor saccharolactone. The ether-soluble material released was chromatographed on Celite in benzene:hexane:70% methanol (55:45:100; ref. 8) and the E_2-containing fraction crystallized with carrier E_2, before and after acetylation, to constant SA and, where appropriate, $^3H/^{14}C$ ratio. Aliquots of chromatographic peaks containing $E_2$17G were hydrolyzed and chromatographed as above in order to identify the labeled aglycone. The greater part of each $E_2$17G-containing peak was methylated (7) and then hydrolyzed (7) and the resulting ether-soluble radioactivity was crystallized with carrier $E_2$3MeE to constant SA (and, where appropriate, isotope ratio) prior to acetylation and recrystallization (7). Melting points of final derivatives were checked on a microscope hot stage. Peaks containing E_1 glucosiduronate were hydrolyzed and then chromatographed to identify the aglycone estrone.

All radioactive counting was performed as described elsewhere (4, 11) except that direct counting of urine, and aqueous samples from DEAE-Sephadex columns, was carried out after mixing directly with Aquasol (a xylene-based scintillation fluid purchased from NENC).

Results

General. The presence of saccharolactone effectively inhibited the β-glucuronidase hydrolysis of separated conjugate fractions. In the absence of the inhibitor, and when ^{14}C-$E_2$3G and/or ^{14}C-$E_2$17G had been added as internal standards, release of ^{14}C in an ether-soluble form was at least 95% under the hydrolytic conditions employed. Hydrolytic release of ^3H varied throughout the time period studied, presumably due to the presence of metabolites other than glucosiduronates of E_1 and E_2, which metabolites were not hydrolyzed completely by 500 U/ml of Ketodase for 20 hours. During each chromatographic step, including Amberlite XAD-2 treatment to recover the conjugates from aqueous solution following each DEAE-Sephadex chromatography, no more than 5 to 10% of each ^{14}C-standard was lost.

Intravenous ^3H-E_2. Within two hours after injection, three well-defined peaks of ^3H were separable by DEAE-Sephadex chromatography (Fig. 1 and 2). Peak I was at least 90% E_1 glucosiduronate, peak II corresponded with standard ^{14}C-$E_2$3G (Fig. 1, subject CR), while peak III corresponded

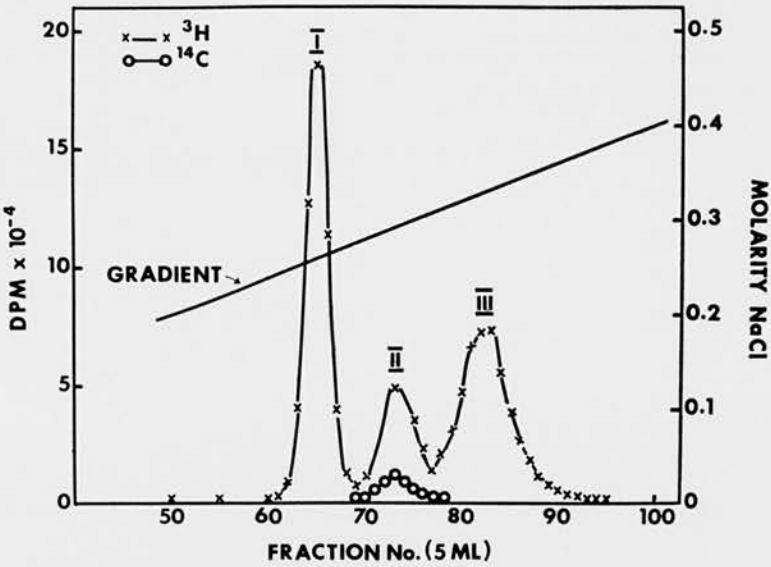

FIG. 1. Final DEAE-Sephadex chromatography of urinary ^{3}H-glucosiduronates (0–2 hr) after iv injection of ^{3}H-E_2 into subject CR. Pure ^{14}C-E_2 3G was added prior to chromatography.

with added ^{14}C-$E_2$17G (Fig. 2, subject DE). Table 1 provides further evidence for the presence of ^{3}H-$E_2$3G (approx. 55% of total ^{3}H in the peak) in peak II. Table 2 shows similar evidence in favor of ^{3}H-$E_2$17G being virtually the sole ^{3}H-constituent of peak III. In these two experiments, over zero to two hours, the ratio $E_2$17G: $E_2$3G = 4:1 and 4.5:1, respectively.

At times between two and 24 hours following injection, only two well-defined ^{3}H-peaks could be seen after DEAE-Sephadex chromatography (Fig. 3, subject DE, 2–4 hr), these corresponding to peaks I and II of Fig. 1 and 2. In all instances beyond two hours peak II was shown to contain ^{3}H-$E_2$3G, which, as a percentage of the total peak, varied with

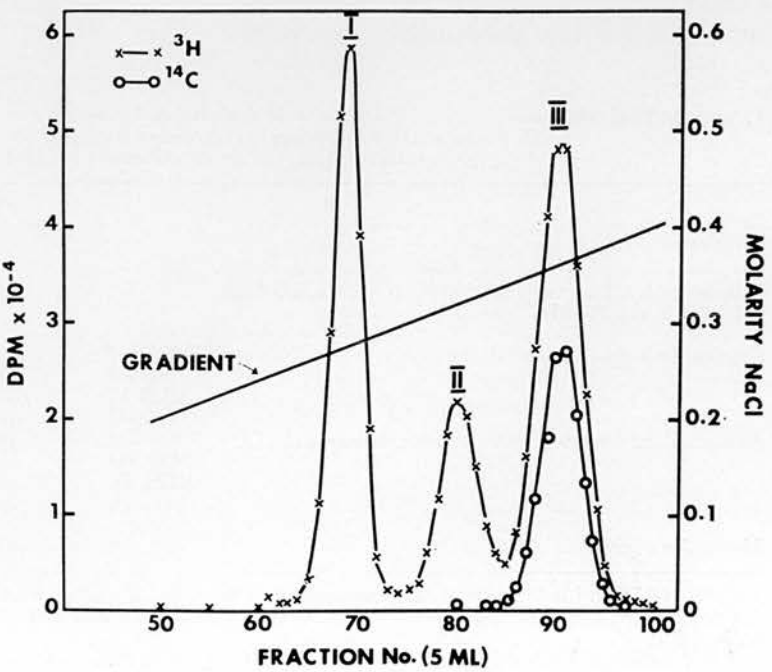

FIG. 2. Final DEAE-Sephadex chromatography of urinary ^{3}H-glucosiduronates (0–2 hr) after iv injection of ^{3}H-E_2 into subject DE. Pure ^{14}C-E_2 17G was added prior to chromatography.

TABLE 1. Identification of ^3H-E_2 as an aglycone released by enzyme hydrolysis of peak II (from a DEAE-Sephadex chromatogram of the 0–2 hr urine, subject CR,* after iv injection of ^3H-E_2)

Purification step		^3H SA (dpm/mg)	^{14}C SA (dpm/mg)	$^3H/^{14}C$
Aliquot of E_2 fraction following CPC; 91,300 dpm ^3H, 24,100 dpm ^{14}C +36.9 mg carrier E_2		2470 (calculated)	653	3.8
Crystallization from methanol	XLS I	2350	648	3.6
	ML I	2650	680	3.9
	XLS II	2230	662	3.4
	ML II	2410	674	3.6
Acetylation; crystallization from methanol-H_2O†	XLS III	2350	628	3.7
	ML III	2410	650	3.7
	XLS IV	2410	670	3.6
	ML IV	2380	658	3.6
Mean for crystals		2330	652	3.6

* ^{14}C-E_2 3G added prior to chromatography (see Fig. 1); after final DEAE-Sephadex column $^3H/^{14}C$ for peak II = 6.1; after enzyme hydrolysis + extraction, ratio = 5.1; after CPC → E_2 fraction, ratio = 3.8
† SA values calculated for free steroid (XLS = crystals, ML = mother liquor).

time and was considerably less in this respect than the 55% quoted for zero to two hours. This percentage decrease was related to the appearance of additional ^3H-metabolites, such as estriol glucosiduronate, in peak II. Peak I contained E_1 glucosiduronate, again as a varying percentage, over the period studied. The chromatographic separation between peaks I and II tended to become less distinct with time (see Fig. 3), presumably due to the increasing amounts of additional metabolites. Attempts to verify whether small amounts of ^3H-$E_2$17G might be present beyond two hours led to the conclusion that of the total ^3H-$E_2$17G in the urine within 24 hours some 96% was in zero to two hours, no more than 3% was in two to four hours and <1% was in four to six hours.

Oral ^3H-E_2. In both experiments th

TABLE 2. Identification of ^3H-$E_2$3MeE as the derivative formed by methylation and hydrolysis of peak III (from a DEAE-Sephadex chromatogram of the 0–2 hr urine, subject DE,* after iv injection of ^3H-E_2)

Purification step		^3H SA (dpm/mg)	^{14}C SA (dpm/mg)	$^3H/^{14}C$
Aliquot of hydrolyzate; 98,800 dpm ^3H, 47,800 dpm ^{14}C +25.3 mg $E_2$3MeE carrier		3900 (calculated)	1890	2.1
Crystallization from methanol	XLS I	3730	1890	2.0
	ML I	4150	1900	2.2
	XLS II	3760	1930	2.0
	ML II	4050	1960	2.1
Acetylation; crystallization from methanol-H_2O†	XLS III	3850	1890	2.0
	ML III	4010	1920	2.1
	XLS IV	3760	1930	2.0
	ML IV	3850	1940	2.0
Mean for crystals		3780	1910	2.0

* ^{14}C-$E_2$17G added prior to chromatography (see Fig. 2); after final DEAE-Sephadex column $^3H/^{14}C$ for peak III = 2.2; after methylation and extraction, ratio = 2.2; after enzyme hydrolysis and extraction ratio = 2.1.
† SA values calculated for free steroid (XLS = crystals, ML = mother liquor).

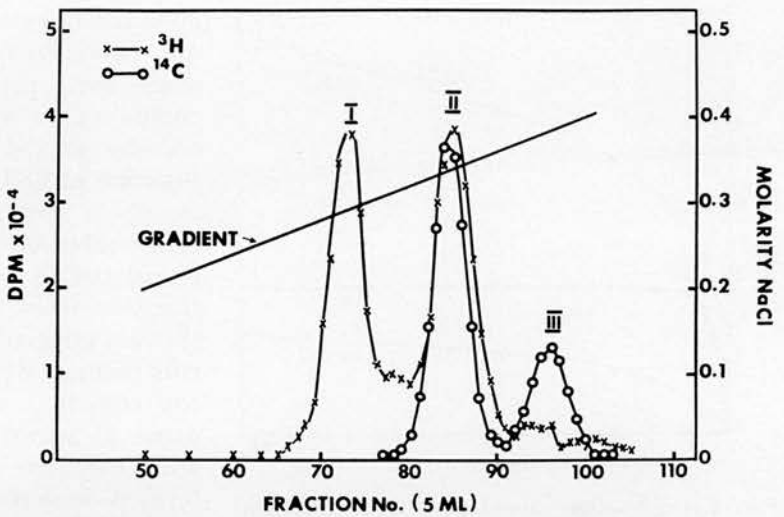

FIG. 3. Final DEAE-Sephadex chromatography of urinary ^{3}H-glucosiduronates (2–4 hr) after iv injection of ^{3}H-E_2 into subject DE. Pure ^{14}C-E_2 3G and ^{14}C-$E_2$17G were added prior to chromatography.

DEAE-Sephadex chromatographic pattern over the period zero to 24 hours was that shown in Fig. 4 (0–2 hr, subject JS). Two ^{3}H-peaks were found, these corresponding in position to peaks I and II described above. Peak I contained E_1 glucosiduronate at each time period, while peak II was shown to contain ^{3}H-E_2 3G. At any given time, ^{3}H-$E_2$3G, as a percentage of total ^{3}H in peak II, was greater following ingestion than after intravenous administration. Thus, after oral administration about 85% of peak II in zero to two hours was made up of $E_2$3G, this value decreasing in later urine samples. In neither oral study was there any significant amount of ^{3}H, associated with peak III, which could be identified as ^{3}H-$E_2$17G.

Cumulative excretion of E_2 monoglucosiduronates. Fig. 5 shows the cumulative excretion of ^{3}H-$E_2$3G after intravenous and oral ^{3}H-E_2, and of ^{3}H-$E_2$17G after intravenous ^{3}H-E_2, over a zero to 24 hour period. For the purposes of comparison these values have been calculated relative

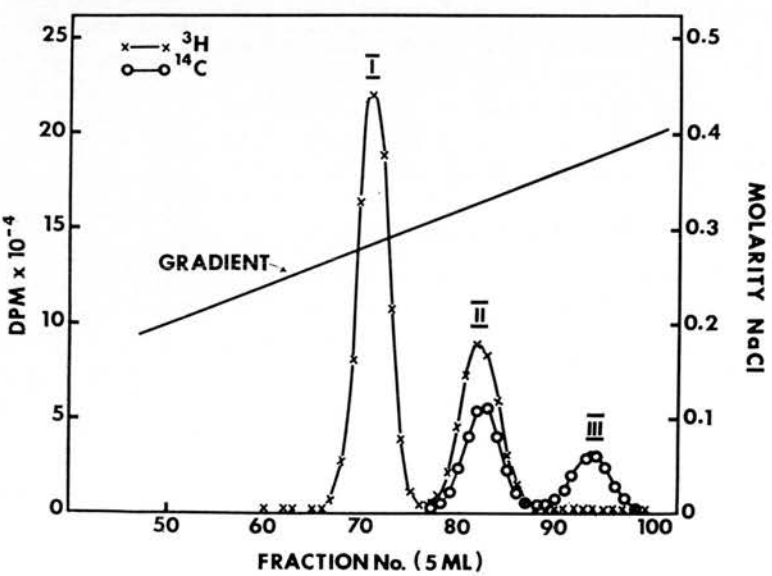

FIG. 4. Final DEAE-Sephadex chromatography of urinary ^{3}H-glucosiduronates (0–2 hr) after oral administration of ^{3}H-E_2 to subject JS. Pure ^{14}C-$E_2$3G and ^{14}C-$E_2$17G were added to the urine prior to the purification procedure.

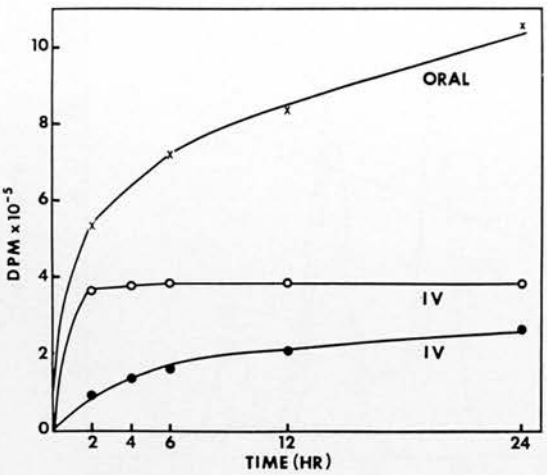

FIG. 5. Cumulative excretion of ^{3}H-E_2 monoglucosiduronates: x—x = E_2 3G from oral E_2 (subject JN); ○—○ = E_2 17G from iv E_2 (average of 2 exp); ●—● = E_2 3G from iv E_2 (average of 2 exp).

to a dose of 40×10^6 dpm ^{3}H-E_2 in each experiment. The data represent the mathematical products of the various crystallized fractions and the corresponding masses of carrier steroids added prior to crystallization. No correction was made for normal procedural losses during the various chromatographic steps. It can be seen that the early urinary production of $E_2$17G from circulating E_2 was greatly in excess of $E_2$3G but, whereas the latter continued to be excreted up to (and presumably beyond) 24 hours, the former effectively ceased after two to four hours. The rate of excretion of $E_2$3G after ingestion of E_2 appeared to be greater than after intravenous injection of E_2.

Discussion

It seems clear from this study that both $E_2$17G and $E_2$3G are metabolites of intravenously administered E_2 in the normal human female. The early occurrence of these two conjugates in the urine rather indicates a direct glucuronylation of E_2, favoring conjugation at C-17 over that at C-3, perhaps in the liver. The time relationship would certainly appear to exclude an enterohepatic formation (12). This does not, however, rule out the possibility that some such intermediate as E_2-3-sulfate could act as substrate for a glucuronyl transfer to C-17, with subsequent deconjugation at C-3 to yield $E_2$17G. Also, the excretion pattern of the two monoglucosiduronates in question could conceivably be related to a different rate of release of the two from their tissues of origin, although no good evidence is available on this point. Regardless of these factors the cessation of $E_2$17G excretion in the urine at about two hours is compatible with the view that this compound, once formed, is in part rapidly excreted as such in the urine, and partly in the bile in the form of highly polar conjugates such as E_2-3-sulfate-17-glucosiduronate (E_2 3S17G ref. 13; Musey and Hobkirk, unpublished results). It is known that intravenously administered 6,7-^{3}H-$E_2$3G-^{14}C leads to a rapid urinary excretion of radioactivity in the urine (70% of each isotope dose in 0–3 hr, about a further 10% of each in 3–6 hr and a further 10% of ^{3}H and no ^{14}C in approx. 6–48 hr; ref. 11). In the present study it seems likely, therefore, that the early appearance of ^{3}H-$E_2$3G in the urine following intravenous ^{3}H-E_2 largely represents that part of the labeled E_2 which had been converted directly to that monoconjugate, and/or that arising directly from $E_1$3G (11). The continuing excretion of significant amounts of $E_2$3G, however, (see Fig. 5) up to at least 24 hours, could most probably reflect excretion of $E_2$3S17G (and perhaps other conjugates) in the bile with subsequent deconjugation, and reconjugation partly in the form of E_2 3G (ref. 13; Musey and Hobkirk, unpublished results). In support of this is the finding that oral ^{3}H-$E_2$17G results in the urinary excretion of little other than labeled $E_1$3G

[3] Assuming that the metabolism of intravenous $E_2$17G is similar to that of $E_2$17G formed from intravenous E_2.

and $E_2$3G (14), and that intravenously administered ^{3}H-$E_2$17G is considerably converted to urinary metabolites including $E_1$3G, $E_2$3G and $E_2$3S17G (7). Thus, urinary $E_2$3G may be from at least three sources, namely: a) $E_2 \rightarrow E_2$3G, b) $E_2 \rightarrow E_2$17G $\rightarrow E_2$3S17G $\rightarrow E_2$3G, and c) a direct conversion from $E_1$3G (11). This information further complicates the picture when the "total urinary E_2 glucosiduronate" fraction is used as a "metabolite" for calculation of secretion rate of estrogen. The apparent absence of urinary $E_2$17G beyond about four hours after intravenous E_2, and our failure to definitely identify it following oral E_2, suggests that this urinary monoglucosiduronate is not of enteric origin in the human female.

It is interesting to note certain similarities between the metabolism of E_2 monoglucosiduronates and those of estriol (E_3; ref. 15, 16). Thus, $E_3$3G is mainly, if not entirely, of enteric origin, whereas $E_3$16G appears to be formed by both liver and intestine. Also, $E_3$3S16G is the principal biliary metabolite of E_3 and is converted largely to monoglucosiduronates of E_3 in the intestine.

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The Role of an Enterohepatic System in the Metabolism of 17β-Estradiol-17-Glucosiduronate in the Human Female*

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ABSTRACT. 17β-Estradiol-6,7^{3}H-17-glucosiduronate ($E_2$17G) was injected iv in a single dose into 3 normal women and 3 women with biliary drainage via T-tube. Urine and, where applicable, bile was collected over various periods of time and the conjugated metabolites were analyzed by Amberlite resin, DEAE-Sephadex, Celite and thin-layer chromatography, together with selective enzymatic hydrolysis, derivative formation and crystallization with unlabeled carriers. In the normal women 62–70% of the dose appeared in the urine in 24 hr with >80% in 72 hr. Some 95% of the urinary ^{3}H within 2–3 hr after injection (17–48% dose) was $E_2$17G and the remainder was 17β-estradiol-3-sulfate-17-glucosiduronate ($E_2$3S17G). Over about 2–6 hr a lag in ^{3}H excretion occurred, the latter 2 conjugates virtually disappeared and estrone-3-glucosiduronate ($E_1$3G) plus estradiol-3-glucosiduronate ($E_2$3G) appeared in the urine. Beyond 6 hr $E_1$3G and $E_2$3G increased cumulatively and were by far the main metabolites up to 72 hr. No $E_2$17G was excreted at these later times but very small amounts of other glucosiduronates, *e.g.* those of estriol (E_3) and 16-epiestriol (16-epiE_3), were present. In the T-tube patients $E_2$17G was the major labeled conjugate in the urine within 3 hr, accompanied by small amounts of $E_2$3S17G. Major amounts of $E_2$3S17G appeared in the bile unaccompanied by $E_2$17G. An unidentified conjugate, probably a sulfoglucosiduronate, also appeared in variable amounts in the bile. Beyond 3–6 hr the T-tube patients excreted variable amounts of $E_1$3G and $E_2$3G in the urine, these probably arising from biliary $E_2$3S17G which had bypassed the collection tube, and/or from precursors transported into the intestine via the succus entericus. Administration of ^{3}H-$E_2$3S17G directly into the duodenum of a normal female was followed by negligible urinary excretion of ^{3}H up to 6 hr. After that time the urinary metabolite pattern was similar to that seen after 6 hr following iv ^{3}H-$E_2$17G into normal women. Thus the metabolism of $E_2$17G to urinary $E_1$3G and $E_2$3G appears to occur in the intestine from biliary $E_2$3S17G via deconjugation and partial conversion of E_2 to E_1 before or after reconjugation with endogenous glucuronyl groups at C-3. About 7% of the dose was excreted in the bile when labeled $E_1$3G was injected iv into a woman with biliary drainage via T-tube. (*J Clin Endocrinol Metab* **35**: 448, 1972)

COMMUNICATIONS from this laboratory regarding the metabolism of iv administered labeled 17β-estradiol-17-glucosiduronate ($E_2$17G)[4] by the human have already been published (1,2). Although these

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[4] The following trivial names and abbreviations for steroids and their conjugates have been used in the text: 17β-estradiol-17-glucosiduronate ($E_2$17G) = 3-hydroxyestra-1,3,5,(10)-trien-17β-yl-β-D-glucopyranosiduronate; estrone-3-glucosiduronate ($E_1$3G) = 17-oxoestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-3-glucosiduronate ($E_2$3G) = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-3-sulfate-17-glucosiduronate ($E_2$3S17G) = estra-1,3,5(10)-trien-3-yl-sulfate-17β-yl-β-D-glucopyranosiduronate; 17β-estradiol-3,17-diglucosiduronate = estra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate-3-yl-β-D-glucopyranosiduronate; estriol-3-sulfate-16-glucosiduronate ($E_3$3S16G) = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-sulfate-16α-yl-β-D-glucopyranosiduronate; estriol-16-glucosiduronate ($E_3$16G) = 3,17β-dihydroxyestra-1,3,5(10)-trien-16α-yl-β-D-glucopyranosiduronate; 2-

studies showed conclusively that $E_2$17G is metabolized, mainly to urinary estrone-3-glucosiduronate ($E_1$3G) and 17β-estradiol-3-glucosiduronate ($E_2$3G), together with small amounts of 17β-estradiol-3-sulfate-17-glucosiduronate ($E_2$3S17G), the sequence of formation of the metabolites, and hence the metabolic pathways involved, was not established. $E_2$17G was found in the labeled form in the urine, probably representing unchanged injected material. Since only minor amounts of urinary conjugates other than those of 17β-estradiol (E_2) and estrone (E_1) were identified in these experiments it was concluded that metabolism did not proceed via free E_2 (at least not via liberation of E_2 into the main body pool) but rather, perhaps through some conjugated intermediate. In order to elucidate these matters in the present work, ^{3}H-$E_2$17G has been injected iv into normal female volunteers, into female volunteers draining bile via T-tube, and ^{3}H-$E_2$3S17G has also been instilled into the duodenum of one normal female volunteer. In addition, in order to enquire further into the excretion of monoglucosiduronates in the bile, estrone-6,7-^{3}H-3-glucosiduronate-^{14}C (^{3}H-$E_1$3G-^{14}C) was injected intravenously into one female volunteer draining bile via a T-tube. Urinary and biliary metabolites were studied at various time intervals. An abstract of part of this work has appeared (3).

Materials and Methods

General. All chemicals and other reagents, including organic solvents, unlabeled steroids, enzymes and chromatographic materials, were obtained, purified when necessary, and used, a described in previous publications (4–7).

Labeled steroid conjugates. ^{3}H-$E_2$17G of SA = 2.2 μCi/μg was purchased from New Englan Nuclear Corp., Boston, Mass. (NENC) and wa purified by published methods (1,4). E_2-4-^{14}C 17G (^{14}C-$E_2$17G) was synthesized by NEN (7) while E_1-4-^{14}C-3G (^{14}C-$E_1$3G), E_2-4-^{14}C-3 (^{14}C-$E_2$3G), E_1-4-^{14}C-3-sulfate (^{14}C-$E_1$3S) an E_2-4-^{14}C-3-sulfate (^{14}C-$E_2$3S) were prepared i our laboratory (5). These latter 5 conjugate were employed as internal standards at variou points in the experimental procedures. ^{3}H $E_2$3S17G was prepared by incubating ^{3}H-$E_2$17 with a 105,000 × *g* supernatant from femal guinea pig liver in the presence of ATP, MgSC and KCl (5). ^{3}H-$E_1$3G-^{14}C (glucuronic aci generally labeled) was prepared as already de scribed (8) by mixing appropriate quantities c E_1-6,7-^{3}H-3G (^{3}H-$E_1$3G)—prepared by incu bating guinea pig liver homogenate with ^{3}H-E (NENC, SA = 10 μCi/μg) and unlabeled uri dine diphosphate glucuronic acid (UDPGA)— with E_1-3G-^{14}C prepared by a similar incuba tion involving unlabeled E_1 and UDPGA-^{14} (NENC, SA = 212 μCi/μg). After purificatio (8) the ^{3}H-$E_1$3G-^{14}C had a ^{3}H/^{14}C ratio of 5.7

Administration of labeled conjugates; collectio of bile and urine. Prior to administration eac compound was filtered through Swinnex-13 fil ters of pore size 22 μ (Millipore Corp., Bedford Mass.).

^{3}H-$E_2$17G was injected in a single dose int an arm vein of each of three normal females a about day 10 of the menstrual cycle, as de scribed previously (6). Subject JJ, aged 34 yr received 12.9 × 10^6 dpm; subject EN, 27 yr received 54.9 × 10^6 dpm; subject TL, 25 yr received 41.9 × 10^6 dpm of ^{3}H. Urine collec tions were made from JJ at 0–6, 6–12, 12–24 24–48 and 48–72 hr; from EN at similar time except that 0–6 hr was divided into 0–3 an 3–6 hr; from TL at 0–2, 2–4, 4–6, 6–12, an 12–24 hr.

^{3}H-$E_2$17G was injected as above into thre females who had undergone cholecystectomy fo gallstones about 7 days previously and who wer draining bile via T-tube. At the time of injec tion all liver function tests were in the norma range. Bile was collected from each subject ove 0–3, 3–6 and 6–24 hr while urine was collecte

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methoxyestrone (2-MeOE_1) = 2,3-dihydroxestra-1, 3,5(10)-trien-17-one-2-methyl ether; 16-epiestriol (16-epiE_3) = estra-1,3,5(10)-trien-3,16β,17β-triol; ring Dα-ketols = 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one + 3,16β-dihydroxyestra-1,3,5(10)-trien-17-one + 3,17β-dihydroxyestra-1,3,5(10)-trien-16-one; 2-methoxyestradiol-17β = estra-1,3,5(10)-trien-2,3,17-triol-2-methyl ether; 17α-estradiol = estra-1, 3,5(10)-trien-3,17α-diol; dihydroequilin = estra-1,3, 5(10), 7(8)-tetraen-3,17β-diol; dihydroequilenin = estra-1,3,5(10), 6,8(9)-pentaen-3,17β-diol.

over 0–3, 3–6, 6–12 and 12–24 hr. Each subject, MT-31 yr, SA-36 yr and LM-31 yr, received 48 × 10^6 dpm of ^{3}H.

^{3}H-$E_2$3S17G (42.7 × 10^6 dpm) was instilled in 10% (v/v) ethanol-H_2O followed by H_2O washings, into the second portion of the duodenum (confirmed by X-ray) of normal female JL (25 yr). Urine was collected over 0–3, 3–6, 6–12, 12–24, 24–48, 48–72, and 72–96 hr.

^{3}H-$E_1$3G-^{14}C (12.6 × 10^6 dpm ^{3}H, 2.21 × 10^6 dpm ^{14}C) was injected iv, as above, in a single dose, into an arm vein of female subject MM (25 yr) who was draining bile via T-tube, having undergone cholecystectomy for gallstones 7 days previously. All liver function tests were normal. Bile and urine were collected over 0–3, 3–6, 6–12 and 12–24 hr.

All urine and bile samples were frozen (—15 C) until analyzed.

Analysis of urinary metabolites. The methodology has already been described in some detail (2,4,7,8). Urine was chromatographed on Amberlite XAD-2 resin (9) and the conjugates eluted were further chromatographed on DEAE-Sephadex in linear concentration gradients of NaCl (0–0.8M and 0–0.4M; ref. 4). Further purification was achieved by Celite partition chromatography (CPC) in iso-octane: *t*-butanol: MNH_4OH (2:5:5, refs. 5, 10) and/or *n*-butanol:ethyl acetate: 0.2% NH_4OH (3:1:4, ref. 11). Radioactive peaks corresponding to $E_1$3G and $E_2$3G were incubated with Ketodase (β-glucuronidase) in the presence and absence of inhibitor saccharo-1,4-lactone (7) and the aglycones identified by a combination of Girard separation and CPC (12) then finally by crystallization to constant SA with appropriate carrier steroids before and after acetylation (6). In some instances $E_1$3G peaks were reduced with $NaBH_4$ (8) prior to enzyme hydrolysis and the aglycone was identified as E_2 by the above methods. On occasion 3H-labeled $E_1$3G and $E_2$3G fractions were rechromatographed in admixture with ^{14}C-$E_1$3G and ^{14}C-$E_2$3G. Labeled peaks corresponding to $E_2$17G were identified by methylation, hydrolysis and crystallization with carrier 17β-estradiol-3-methyl ether ($E_2$3MeE) before and after acetylation (2,7). Aliquots of radioactive peaks corresponding to $E_2$3S17G were incubated separately with Mylase P (to remove the 3-sulfate group; refs. 2,5) and β-glucuronidase (to remove the 17-glucuronyl group; refs. 2,3) and the products, $E_2$17G and $E_2$3S respectively, were chromatographed on DEAE-Sephadex and/or processed by CPC in admixture with ^{14}C-$E_2$17G and ^{14}C-$E_2$3S. Each was then further identified by hydrolysis (Mylase P incubation of $E_2$3S; β-glucuronidase incubation of $E_2$17G before or after methylation; refs. 2,5) followed by crystallization of the steroid moiety as above. Conjugates of steroids other than E_1 and E_2, *e.g.* estriol (E_3), 2-methoxyestrone (2-MeOE_1) and 16-epiestriol (16-epiE_3), were sought, through a combination of Girard separation and CPC (6) and in some cases by crystallization.

Analysis of biliary metabolites. Total biliary radioactivity was measured in 0.1 ml volumes of bile in a dioxane-based liquid scintillation medium (6) or, later in the study, in a xylene-based medium (Aquasol, NENC, ref. 7). Initial attempts to recover biliary conjugates by passing diluted bile (1:5 with H_2O) through XAD-2 resin were generally not satisfactory. Small volumes of bile (up to 15 ml) could, however, be directly chromatographed on DEAE-Sephadex in a linear concentration gradient (0–0.8M) of NaCl (4,5). Although such columns ran very slowly the eluted conjugates were well separated from the main pigments and could subsequently be rechromatographed on DEAE-Sephadex or by CPC with some ease. Identification of biliary conjugates was attempted in the general fashion described for urinary conjugates (2,4–8).

All of the radioactive counting was performed using a Nuclear Chicago liquid scintillation spectrometer (Model 6725) and in a manner previously described (6–8).

Results

Urinary metabolites of iv ^{3}H-$E_2$17G in normal subjects. As shown in Table 1 a sizable amount of ^{3}H was excreted, apparently within 2 or 3 hr followed by a lag period, up to between 4 and 6 hr during which very little isotope was excreted. After about 6 hr the excretion recommenced and continued up to at least 72 hr. In the three subjects 62–70% of the dose was excreted in 24 hr while more than 80% was excreted in 72 hr

TABLE 1. Urinary excretion of radioactivity (% dose) after iv injection of ^{3}H-$E_2$17G into normal women

Time following injection (hr)	Subjects		
	JJ	EN	TL
0–2		20 (0–3)*	43
2–4	28 (0–6)*		3.4
4–6		1.6 (3–6)*	1.5
6–12	20	20	10
12–24	22	20	10
24–48	10	15	**
48–72	7.5	5.6	**
Total	88	82	68 (24 hr)

* Specific periods of urine collection.
** Not studied.

by the two subjects from whom urine was collected for that length of time.

Within 2 hr (TL) and 3 hr (EN) about 95% of the urinary ^{3}H was identified as $E_2$17G, presumably unchanged injected material, the remainder being $E_2$3S17G. At some point beyond 2 hr and within 6 hr the latter two compounds declined and virtually disappeared while $E_1$3G and $E_2$3G appeared and exhibited a cumulative increase with time. The 0–6 hr urine from JJ contained mainly $E_2$17G and small amounts of $E_2$3S17G but also the early portion of $E_1$3G and $E_2$3G. In JJ and EN no $E_2$17G or $E_2$3S17G was detected beyond 6 hr. In TL 1% of the dose appeared in the urine as $E_2$17G in 6–12 and 12–24 hr, while $E_2$3S17G (no more than 0.3% of the dose) was found in each of these time intervals. Fig. 1 shows the cumulative excretion pattern of metabolites for subject EN while Table 2 contains cumulative totals for the identified E_1 and E_2 conjugates in the three subjects. The widely varying excretion of ^{3}H-$E_2$17G (17–48% dose) should be noted as should the constant ratio $E_1$3G: $E_2$3G of 2.3, 2.4 and 2.2 in JJ, EN and TL, respectively. Beyond 12 hr following injection additional ^{3}H-glucosiduronates (*e.g.* E_3 and 16-epiE_3) were detected in very small amounts. In these experiments the urinary ^{3}H was almost completely identified as conjugates of E_1 and E_2. Losses during

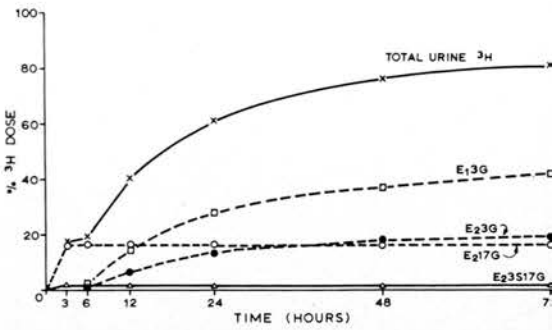


FIG. 1. Cumulative urinary excretion of total ^{3}H and ^{3}H-labeled metabolites after iv injection of ^{3}H-$E_2$17G into subject EN.

purification were typical of normal methodology and were reflected in small losses of ^{14}C-$E_1$3G and ^{14}C-$E_2$3G added as internal standards.

^{3}H in urine and bile after iv ^{3}H-$E_2$17G in subjects with T-tube drainage. A variable percentage of the dose appeared in bile and urine (Table 3). Total recoveries in MT, SA and LM were 99, 86 and 87%, respectively. Biliary recovery was 28–65% and urinary recovery was 21–59%.

Urinary metabolites of iv ^{3}H-$E_2$17G in T-tube subjects. Over 0–3 hr 78–95% of urinary ^{3}H was identified as $E_2$17G and the

TABLE 2. Cumulative total urinary metabolites (% dose) of iv injected ^{3}H-$E_2$17G into normal women

Metabolites	Subjects		
	JJ* (up to 72 hr)	EN* (up to 72 hr)	TL** (up to 24 hr)
$E_1$3G	42	45	11
$E_2$3G	18	19	5
$E_2$17G	24	17	48
$E_2$3S17G	1.3	1	3
Total	85	82	67

* Very small amounts of additional glucosiduronates were detected by CPC: *e.g.*, 16-epiE_3, E_3 2-MeOE_1, particularly after 24 hr.

** E_3 glucosiduronate (0.2% dose) was identified by crystallization of the aglycone in 12–24 hr. 16-EpiE_3 glucosiduronate (0.1% dose) was detected by CPC in the same time period.

TABLE 3. Excretion of ^3H (% dose) in urine and bile after iv injection of ^3H-$E_2$17G into 3 females draining bile via T-tube

Time interval (hr)	Subjects					
	MT		SA		LM	
	Urine	Bile	Urine	Bile	Urine	Bile
0–3	30	40	15	48	19	11
3–6	2	2	2	10	18	2.6
6–12	6		1		18	
		7*		7*		14*
12–24	12		3		4	
Total	50	49	21	65	59	28

* 6–24-hr collection.

remainder as $E_2$3S17G in the three subjects. Between 3 and 6 hr the small amount of ^3H (2% dose in urine) in MT and SA consisted of $E_1$3G and $E_2$3G, together with minute amounts of $E_2$3S17G in MT, and residual $E_2$17G and $E_2$3S17G together with traces of an unknown sulfoglucosiduronate conjugate termed, for present purposes, E-SG (see below for bilary conjugates), in SA. The larger fraction of the dose (18%) excreted in 3–6 hr by LM consisted mainly of $E_1$3G and $E_2$3G with smaller amounts of $E_2$17G and traces of $E_2$3S17G. Between 6 and 24 hr after injection virtually all the urinary ^3H in MT was $E_1$3G and $E_2$3G, while very small amounts of E_3 and possibly ring Dα-ketols were detected at 12–24 hr in this subject after β-glucuronidase hydrolysis. A similar predominance of $E_1$3G and $E_2$3G occurred over the same period in SA and LM. In both the latter subjects E-SG was detected and in MT small but definite amounts of $E_2$17G and $E_2$3S17G persisted up to 12–24 hr. Fig. 2 shows the cumulative excretion pattern of the urinary metabolites for subject MT. Table 4 contains cumulative totals for the metabolites at 24 hr in the three experiments. $E_1$3G and $E_2$3G together were highly variable, accounting for 35, 18 and 2.4% of the ^3H-$E_2$17G dose in LM, MT and SA, respectively.

Biliary metabolites of iv ^3H-$E_2$17G. Fig. 3 (subject MT) is qualitatively representative

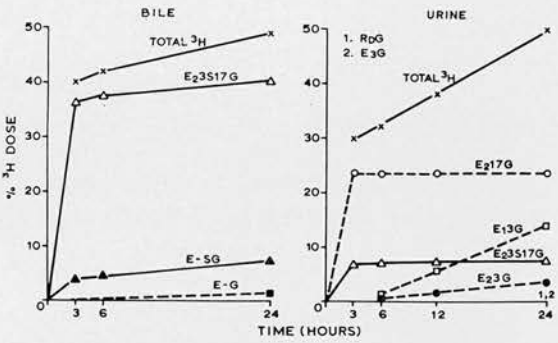


FIG. 2. Cumulative excretion of labeled metabolites of iv injected ^3H-$E_2$17G in the urine and bile of subject MT. R_D-G = "ring Dα-ketols" detected by CPC after β-glucuronidase hydrolysis.

of the DEAE-Sephadex patterns (0–0.8M NaCl) obtained for 0–3 hr bile samples of the three subjects. The 3–6 and 6–24 hr samples also appeared to be substantially in this form. In addition, the 6–24 hr sample from MT contained a monoglucosiduronate of an unidentified aglycone (1.5% of the dose and termed here E-G), while the 0–3 hr sample from SA contained a monosulfate (1.6% of the dose, termed here E-S). The

TABLE 4. Total cumulative excretion at 24 hr (% dose) in urine and bile of ^3H-metabolites after iv injection of ^3H-$E_2$17G into 3 females draining bile via T-tube

Metabolites	Subjects					
	MT*		SA		LM	
	Urine	Bile	Urine	Bile	Urine	Bile
$E_1$3G	14	—	2.2	—	28	—
$E_2$3G	3.5	—	0.2	—	7	—
$E_2$17G	23	—	17	—	18	—
$E_2$3S17G	7	40	1	29	1.7	14
E-SG	—	7.4	0.5	34	0.7	7
E-G	—	1.5	—	—	—	—
E-S	—	—	—	1.6	—	—
E_3G	0.5	—	—	—	—	—
ring Dα-ketols	0.8**	—	—	—	—	—
Total	49	49	21	65	55	21

* Small amounts of 16-epi$_3$ and 2-MeOE_1 were detected by CPC after β-glucuronidase hydrolysis.

** Detected by CPC after β-glucuronidase hydrolysis.

second labeled peak in Fig. 3 was identified as $E_2$3S17G in all cases. The first labeled peak (Fig. 3) behaved on hydrolysis as a sulfoglucosiduronate conjugate but the "steroidal moiety" has thus far escaped identification. It is termed E-SG in this paper since E is possibly the "steroid" present in E-G and E-S, above. Biliary E-SG was 7, 34 and 7% of the dose in MT, SA and LM, respectively, whereas the corresponding values for $E_2$3S17G were 40, 29 and 14%. Cumulative patterns of biliary metabolites are shown for MT in Fig. 2 and cumulative totals, at 24 hr, are shown for the three subjects in Table 4.

Attempts to identify E-SG. Incubation with phenolsulfatase (Mylase P) yielded a ^{3}H-labeled compound (ether-insoluble) which on DEAE-Sephadex (0–0.8M NaCl) appeared in fractions 24–32 whereas simultaneously chromatographed ^{14}C-$E_2$17G was eluted in fractions 28–35. β-Glucuronidase treatment of E-SG gave an ether-insoluble product which was eluted in the above system in fractions 49–58, while ^{14}C-$E_1$3S and ^{14}C-$E_2$3S appeared in fractions 57–62 and 63–70, respectively. Complete hydrolysis of E-SG to a non-ketonic ether-extractable form was achieved by sequential incubation with the above two enzyme preparations. CPC of this ^{3}H- "steroid" in benzene: hexane:methanol:H_2O (55:45:70:30) resulted in elution of the label in fractions 5–9. ^{3}H-17α-estradiol[5] was eluted in fractions 9–12 and ^{14}C-E_2 in 10–14. Thin-layer chromatography of "E" in ethyl acetate: cyclohexane (1:1, ref. 13) suggested a similarity to dihydroequilin or dihydroequilenin. However, crystallization with the unlabeled forms of these[6] and with a number of other carriers, including 2-methoxyestradiol-17β, did not result in the achievement of radiochemical homogeneity.

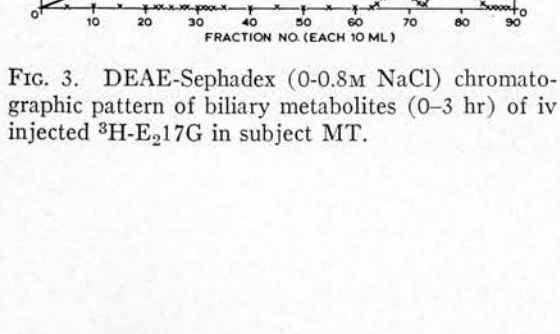


FIG. 3. DEAE-Sephadex (0-0.8M NaCl) chromatographic pattern of biliary metabolites (0–3 hr) of iv injected ^{3}H-$E_2$17G in subject MT.

TABLE 5. Urinary excretion of ^{3}H after intraduodenal administration of ^{3}H-$E_2$3S17G to normal female JL

Time interval (hr)	Urinary ^{3}H (% dose)
0–3	0.1
3–6	0.2
6–12	14.7
12–24	12.4
24–28	19
48–72	15
72–96	9
Total	70

Urinary metabolites of intraduodenal ^{3}H-$E_2$3S17G. The ^{3}H excretion pattern is shown in Table 5. A very minute amount of label appearing in 0–3 hr was tentatively identified as $E_2$3S17G by enzymatic hydrolysis and CPC. Beyond 6 hr the increased excretion was largely accounted for as labeled $E_1$3G and $E_2$3G with smaller quantities of E_3, 16-epiE_3 and 2-MeOE_1 which were released by β-glucuronidase and crystallized with carriers. Fig. 4 shows the cumulative excretion pattern of the various metabolites. Cumulative totals at 72 hr (% dose) were; $E_1$3G = 44, $E_2$3G = 14.4, E_3G = 2.4, 16-epiE_3G = 1.3, 2-MeOE_1G = 1.3 and "ring Dα-ketols G" = 3.1, total = 67%. The

[5] Kindly donated by Dr. D. S. Layne, Dept. of Biochemistry, Faculty of Medicine, University of Ottawa, Ottawa, Ont., Canada.

[6] Kindly donated by Ayerst Laboratories, St. Laurent, Quebec, Canada.

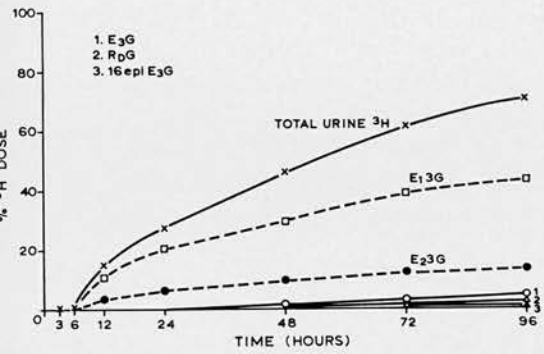


FIG. 4. Cumulative urinary excretion of labeled metabolites of intraduodenally-instilled ^{3}H-$E_2$3S17G in subject JL. R_D-G = "ring Dα-ketols" detected by CPC after β-glucuronidase hydrolysis. See text for metabolite excreted over 0–3 hr.

"ring Dα-ketols" were detected by CPC only.

Urinary and biliary metabolites of iv ^{3}H-$E_1$3G-^{14}C. The excretion pattern of the labels is shown in Fig. 5. A small though measurable amount of radioactivity appeared in the bile while major excretion was evident via the urine. The metabolite patterns in bile and urine, with isotope ratios, are shown in Table 6 for the first 6 hr of this experiment. The presence of both $E_1$3G and $E_2$3G of unchanged $^{3}H/^{14}C$ ratio (compared with that injected) in both bile and urine within 3 hr of injection should be noted.

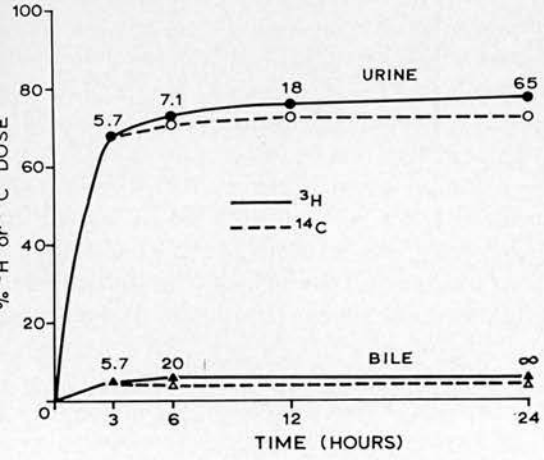


FIG. 5. Cumulative excretion of ^{3}H and ^{14}C in urine and bile of subject MM following iv injection of ^{3}H-$E_1$3G-^{14}C ($^{3}H/^{14}C$ = 5.7). Inscribed numbers refer to $^{3}H/^{14}C$ ratios at the various times.

TABLE 6. Labeled urinary and biliary metabolites excreted within 6 hr of an iv injection of ^{3}H-$E_1$3G-^{14}C ($^{3}H/^{14}C$ = 5.7) into normal female MM

	Urine		Bile	
Metabolites	0–3 hr	3–6 hr	0–3 hr	3–6 hr
$E_1$3G (% ^{3}H dose)	59	4	4	2
$E_1$3G ($^{3}H/^{14}C$)	5.7	6.7	5.7	9.4
$E_2$3G (% ^{3}H dose)	6	1.2	1.7	*
$E_2$3G ($^{3}H/^{14}C$)	5.7	9.3	5.7	∞

* Negligible radioactivity.

Discussion

Since $E_2$17G is now known to be a metabolite of iv injected E_2 (7) the present investigation represents a study on a naturally-occurring compound. In the normal subject the relative amounts of urinary $E_2$17G, $E_1$3G and $E_2$3G formed from labeled $E_2$17G are highly dependent upon the variable fraction of the latter excreted unchanged. A constant infusion of the precursor, rather than a single injection, might have resulted in a different overall quantitative pattern of metabolites (14,15) and would have mirrored, in a more physiological fashion, the fate of conjugate continuously delivered to the tissues of metabolism. The present study presents a more qualitative picture.

The lag in excretion of the label by the normal subjects at about 2–6 hr following intravenous $E_2$17G is obviously due to the delivery of conjugate(s) via the bile to the intestine, largely in the form of $E_2$3S17G. This lag, and the subsequent production of the main metabolites ($E_1$3G and $E_2$3G) after about 6 hr is in agreement with the present data for the metabolism of intraduodenally administered ^{3}H-$E_2$3S17G and also with earlier work (16) on orally administered ^{3}H-$E_2$17G and ^{3}H-$E_1$3G-^{14}C. In the latter study the substrates underwent efficient deconjugation, then reconjugation with

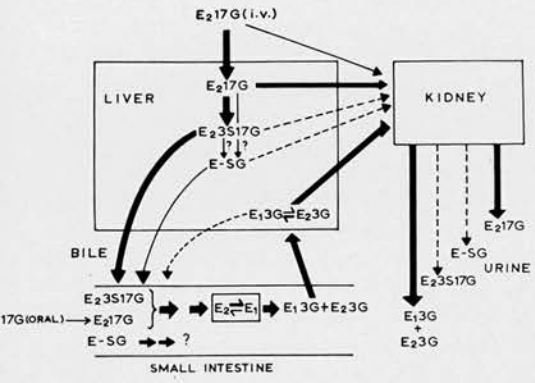
endogenous glucuronyl groups, at the C-3 position only, of E_2 and E_1. Thus it would appear that $E_2$3S17G undergoes a similar fate, with partial conversion of released E_2 to E_1 prior to or after reconjugation with glucuronic acid at C-3. It seems likely that the reconjugation occurs mainly at the intestinal level since considerable absorption of E_2 (or E_1) in the free, or sulfate form would presumably result in formation of major amounts of more highly oxygenated metabolites known to be formed from the free steroids (17). The small amounts of these more polar metabolites (*e.g.* E_3) found in the urine following intravenous $E_2$17G or intraduodenal $E_2$3S17G in the present study could presumably have arisen from precursors (E_1 and/or E_2) absorbed from the intestine in the free or sulfate forms. Note also that direct 16-hydroxylation of $E_1$3G has been observed in the human fetoplacental unit (18). The details of the conversion $E_2$3S17G $\rightarrow$ $E_2$3G + $E_1$3G without the production of major quantities of additional conjugated phenolic steroids is not clear at present but a possible intermediate, speculated upon elsewhere (2), namely, E_2-3, 17-diglucosiduronate, was not detected in this study. It should be noted that a small degree of intestinal absorption of unaltered $E_2$3S-17G may possibly occur based on the presence of a trace of it in the urine following its administration into the duodenum.

The presence of very variable amounts of labeled $E_1$3G and $E_2$3G in the urine of the T-tube subjects at and beyond 3–6 hr following intravenous ^{3}H-$E_2$17G could be due to escape of bile resulting in delivery of some $E_2$3S17G to the intestine.[7] Alternatively, or in part, these metabolites coul also arise from precursors transported int the intestine via the succus entericus, *i.* the enteric circulation of Inoue *et al.* (19 This latter possibility, however, would b unlikely to account for the rather consider able amounts of $E_1$3G and $E_2$3G observed i certain of the subjects.

The metabolism of $E_2$17G bears a resem blance to that of E_3-16-glucosiduronat ($E_3$16G) in the human and some compar ison may be made here. The main biliar metabolite of this conjugate, and of E_3 itsel is E_3-3-sulfate-16-glucosiduronate ($E_3$3S 16G; refs. 11,15,20). Only small amount of the latter appear in the urine since it largely metabolized via deconjugation in th intestine followed by reconjugation as mono glucosiduronates in the intestine and/o liver (21). Both 16- and 3-monoglucosidu ronates of E_3 are formed in the intestine (1 15,22) and the 16- conjugate is also forme in the human liver (11,23). The present re sults support the concept of intestinal fo mation of $E_1$3G and $E_2$3G from intravenou $E_2$17G via biliary $E_2$3S17G. In additio $E_2$3G is the sole urinary E_2 glucosiduronat formed from oral E_2 (7) and it can also ap parently be synthesized from free E_2 at ex tra-enteric sites (7) and from the same pre cursor by human liver *in vitro* (24). More over, urinary $E_2$17G is not an enteric me tabolite of E_2(7) or of $E_2$17G or $E_1$3G (16 in the human, but is formed, possibly intra hepatically, from intravenous E_2 (7).

The apparent absence of $E_2$17G from th bile is of interest and it could be argued tha due to efficient sulfurylation at C-3 little o no unchanged monoglucosiduronate remain to be excreted by this route. However, th

[7] Bile volumes were not sufficiently different between the 3 subjects to indicate widely varying diversion of bile into the duodenum. This could, of course, relate to a variability of total biliary flow between individuals. In MT and SA cholangiograms were performed 20 hr prior to the above experiments and in LM a similar procedure was carried out 3 hr after completion of the experiment. Unimpeded flow of bile was observed in all 3 cases, thus all were *capable* of passing bile into the duodenum at t above times. Therefore the results obtained in th study seem to be compatible with highly variab diversion of bile, and the most likely scheme metabolism for $E_2$17G would be that presented Fig. 6. The possibility of an extra-enteric deconjug tion must also remain as an additional, or alternativ pathway.



IG. 6. Suggested scheme of metabolism for $E_2$17G a the human female. Arrow thickness approximates ne quantitative importance of each step.

mount of unchanged $E_2$17G available for rinary excretion might suggest this to be nvalid. Again, it must be remembered that, ad infusion rather than single injection een carried out, the pattern of biliary me-abolites might have differed. Even so, a mall but definite amount of intravenous $E_1$3G (together with its metabolite, $E_2$3G) ppeared in the bile of the one subject stud-ed. A recent claim has been made[8] that even he latter conjugate is excluded from the ile.

As suggested in an earlier publication (7), ne urinary excretion pattern of E_2 and E_1 nonoglucosiduronates following intravenous H-E_2 appears to agree with the present re-ults. Excretion of $E_2$17G within 2 hr of E_2 njection is complete whereas $E_1$3G and $E_2$3G production continues up to at least 4 hr. The two latter compounds, in all prob-bility, have their origin, at least partly, in iliary $E_2$3S17G. A suggested scheme for ne metabolism of $E_2$17G, based on the pres-nt, and earlier studies from our laboratory 8,16), as well as upon those of others for ne metabolism of E_3 and its conjugates (11, 5,19,21), is shown in Fig. 6. It assumes a major role for liver and intestine in the process but confers upon the kidney only an excretory function.[9]

The occurrence of the unidentified conjugate E-SG in the bile complicates the above picture. Its routes of formation and excretion are not known. Based on the almost complete identification of urinary radioactivity as known metabolites, particularly in the normal subjects, it does not seem likely that important amounts of it, or its metabolites are excreted in the urine. Excretion in the feces would appear possible although no firm data are available. Although E-SG may be on occasion a major biliary metabolite of intravenous $E_2$17G it may not be so with respect to secreted E_2 since $E_2$17G (as judged by urinary studies) is not a major metabolite of the secreted hormone when compared with the amounts of 2- and 16-hydroxy steroids formed from the latter.

Acknowledgments

We are most grateful to Dr. P. R. Blahey, Department of Obstetrics and Gynecology, The Montreal General Hospital, and to Drs. W. R. Ghent (Surgery) and I. T. Beck (Medicine), Hôtel Dieu Hospital, Kingston, for clinical collaboration.

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[9] While the present paper was in the refereeing process Kirdani *et al.* (*J Clin Endocrinol Metab* **34:** 546, 1972) showed the human kidney to be capable of forming E_3-16-glucosiduronate from E_3.

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SECTION H

METABOLISM OF OESTROGEN GLUCOSIDES IN THE HUMAN IN VIVO

METABOLISM OF INTRAVENOUSLY ADMINISTERED 17β-ESTRADIOL-6, 7-^{3}H-3-GLUCOSIDE-^{14}C IN NORMAL WOMEN

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Metabolism of Intravenously Administered 17β-Estradiol-6,7-^{3}H-3-glucoside-^{14}C in Normal Women

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ABSTRACT. 17β-Estradiol-6,7-^{3}H-3-glucoside-^{14}C (uniformly labeled) was prepared and injected intravenously into 2 normal young women. Within about 2 hr 20% of the dose was excreted in the urine of each subject in the form of estrogen glucoside with a substantially unchanged ^{3}H/^{14}C ratio. Approximately 11% of the latter was identified as estrone-3-glucoside, the remainder being the compound injected. ^{3}H-labeled estrogen glucosiduronates and, to a smaller extent, ^{3}H-estrogen sulfates, were also excreted during the first 2 hr and these metabolites continued to appear in the urine, unaccompanied by ^{14}C, up to and including the fourth day after injection. Recovery of ^{3}H in the urine (including that associated with the urinary glucosides) was 60 and 81% in the 2 subjects. The steroid pattern identified in the urinary ^{3}H-glucosiduronate fraction indicated that the major part of the injected 17β-estradiol-3-glucoside had been hydrolyzed, and that the 17β-estradiol released was metabolized in a manner similar to that of intravenously injected 17β-estradiol. (*J Clin Endocr* **32:** 476, 1971)

STEROID conjugation with glucose has not thus far been reported in the human. An estrogen glucoside has, however, been isolated from rabbit urine (1), while others have been made by rabbit tissue *in vitro* (2). An investigation of the metabolism of 17α-estradiol-3-glucoside[3] has also been reported for this species (3). In view of the fact that the metabolism of 17β-estradiol-3-glucosiduronate has been investigated in some detail in the human (4), it was considered worthwhile to study the metabolism of 17β-estradiol-3-glucoside under similar conditions. The study reported here concerns the urinary metabolites of intravenously administered 17β-estradiol-6,7-^{3}H-3-glucoside-^{14}C in the normal human female.

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[1] Research Associate of the Medical Research Council of Canada.

[2] Postdoctoral Fellow of the Medical Research Council of Canada.

[3] The following trivial names for steroids and their conjugates are employed in the text: 17α-estradiol-3-glucoside = 17α-hydroxyestra-1,3,5 (10)-trien-3-yl-β-D-glucopyranoside; 17β-estradiol-3-glucoside = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranoside; estrone-3-glucoside = 17-oxoestra-1,3,5(10)-trien-3-yl-β-D-glucopyranoside; 17β-estradiol-3-glucosiduronate = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; estrone-3-glucosiduronate = 17-oxoestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate; 17β-estradiol-17-glucosiduronate = 3-hydroxyestra-1,3,5 (10)-trien-17β-yl-β-D-glucopyranosiduronate; 2-methoxyestrone = 2-methoxy-3-hydroxyestra-1,3,5(10)-trien-17-one; 16-epiestriol = estra-1,3,5(10)-triene-3,16β,17β-triol; ring D α-ketols = 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one + 3,17β-dihydroxyestra-1,3,5(10)-trien-16-one + 3,16β-dihydroxyestra-1,3,5(10)-trien-17-one.

Materials and Methods

17β-Estradiol-6,7-^{3}H-3-glucosiduronate-^{14}C. This was prepared essentially as described elsewhere (4). Estrone-3-glucosiduronate-^{14}C was synthesized by incubating 0.13 μmole of unlabeled estrone and 50 μCi of uridine diphosphate glucuronic acid-^{14}C (uniformly labeled) of specific activity (SA) 0.173 Ci/mmole (New England Nuclear Corp., Boston, Mass.) with a guinea pig liver homogenate at pH 7.4. A ^{14}C-labeled peak corresponding to estrone-3-glucosiduronate was recovered following DEAE-Sephadex chromatography in 0–0.8M NaCl (4,5) and this was mixed with suitably purified estrone-6,7-^{3}H-3-glucosiduronate (New England Nuclear Corp., SA = 1 Ci/mmole) to yield a ^{3}H/^{14}C ratio of about 6. The mixed material was further purified by DEAE-Sephadex chromatography (0–0.4M NaCl) (5) and by Celite partition chromatography in isooctane:*t*-butanol:M NH_4OH (2:5:5, ref. 4, 7) prior to reduction with $NaBH_4$ (4). The 17β-estradiol-6,7-^{3}H-3-glucosiduronate-^{14}C thus obtained (^{3}H/^{14}C = 5.9) was rechroma-

tographed on DEAE-Sephadex and Celite as above. Hydrolysis with β-glucuronidase confirmed the presence of ^{3}H-17β-estradiol, which was identified by crystallization with carrier steroid followed by derivative formation and recrystallization.

17β-Estradiol-6,7-^{3}H-3-glucoside-^{14}C. This compound was prepared by $NaBH_4$ reduction of the methylester of the 17β-estradiol-6,7-^{3}H-3-glucosiduronate-^{14}C described above. The procedure followed was exactly as described by Collins *et al.* (2) for the preparation of 17α-estradiol-6,7-^{3}H-3-glucoside-^{14}C, except that the amounts of the various reagents employed were reduced to one tenth of those used by these authors. The ratio of ^{3}H to ^{14}C in the 17β-estradiol-6,7-^{3}H-3-glucoside-^{14}C after purification was 5.88.

Reagents, etc. All organic solvents and materials for chromatography were obtained, and purified where necessary, as described elsewhere (8). Ketodase (beef liver β-glucuronidase) was purchased from Warner-Chilcott, Morris Plains, N. J., and was used for hydrolysis in concentrations of 250–500 U/ml at pH 5 and 38 C for times of 20–96 hr. Saccharo-1,4-lactone was purchased from Calbiochem. Ltd., Los Angeles, Cal. Unlabeled steroids were purchased from Mann Research Laboratories, Inc., New York, N. Y. Almond emulsin β-glucosidase and glucono-1,5-lactone were obtained from Sigma Chemical Company, St. Louis, Mo.

Injection of 17β-estradiol-6,7-^{3}H-3-glucoside-^{14}C. Prior to injection the labeled material was filtered through Swinnex-13 filters of pore size 22 μ (Millipore Corp., Bedford, Mass.). Injection (10.6 μCi ^{3}H, 1.8 μCi ^{14}C) was performed in 10 ml of 10% (v/v) ethanol-saline (8) into an arm vein of each of 2 normal 21-yr-old females, MYL and BER, at about day 10 of the cycle. Urine was collected at intervals up to 96 hr (see Results) and frozen until required for analysis.

Analysis of urinary metabolites. Urines from subject MYL over 0–6, 6–24 and 24–48 hr were processed on Amberlite XAD-2 resin (9) followed by DEAE-Sephadex chromatography (0–0.8M NaCl, ref. 6). An eluted peak of radioactivity corresponding to a glucoside conjugate was rechromatographed on Sephadex G25 in H_2O (10) and then on DEAE-Sephadex in H_2O (6). An aliquot of the labeled material recovered was subjected to thin layer chromatography on silica gel H (Merck) in chloroform-ethanol 4:1 and in chloroform-isopropanol-formic acid, 5:3:1. A further aliquot was hydrolyzed with β-glucosidase for 20 hr at 37 C in 0.1M sodium citrate buffer at pH 4.3 (11). This hydrolysis was carried out with and without the presence of glucono-1,5-lactone. The benzene extractable radioactive material obtained by hydrolysis was chromatographed on thin layer plates in benzene-ethyl acetate 7:3.

Peaks from the initial DEAE-Sephadex columns (0–0.8M NaCl), behaving like glucosiduronates, were incubated with Ketodase with and without the addition of saccharolactone. The steroids released were subjected to investigation by Celite partition chromatography following Girard separation (8).

Glucoside-like peaks from subject BER over 0–2 and 2–4 hr were isolated by DEAE-Sephadex chromatography and analyzed as above, as were glucosiduronate-like peaks from the same time intervals. Urine over 4–48 hr was pooled, incubated with Ketodase, and the steroids released identified as above. All radioactive counting was performed as described elsewhere (8).

Results

Table 1 shows the urinary excretion of radioactivity by each subject. In MYL no ^{14}C could be detected beyond 0–6 hr. In BER very little ^{14}C was seen beyond 0–2 hr. The recovery of ^{3}H was considerably greater in BER (81%) than in MYL (60%).

The 0–6 hr urine from MYL yielded, on XAD-2 resin chromatography, 7.18×10^6 dpm ^{3}H and 0.786×10^6 dpm ^{14}C (^{3}H/^{14}C = 9.1). At least 90% of this radioactivity was accounted for following DEAE-Sephadex chromatography (0–0.8M NaCl, collecting 10 ml fractions) which yielded two main broad peaks: peak 1, fractions 5–20 containing 3.94×10^6 dpm ^{3}H and 0.65×10^6 dpm ^{14}C (^{3}H/^{14}C = 6.1), and peak 2, fractions 25–40 containing 2.22×10^6 dpm ^{3}H and no ^{14}C. Some further ^{3}H was associated with the sulfate conjugate fraction but this was not processed. Peak 1 was eluted from Sephadex G25, yielding 3.74×10^6 dpm ^{3}H and 0.595×10^6 dpm ^{14}C (^{3}H/^{14}C = 6.2) in a single peak, and then from DEAE-Sephadex in H_2O to give 3.4×10^6 dpm ^{3}H and 0.573×10^6 dpm ^{14}C (^{3}H/^{14}C = 5.9) also in a single peak. These steps removed considerable amounts of urinary pigments from the fraction. Analysis of this fraction by thin layer chromatography indicated that the

TABLE 1. Excretion of ^{3}H and ^{14}C in the urine (% dose) following injection of 17β-estradiol-6,7-^{3}H-3-glucoside-^{14}C into MYL and BER

MYL			BER		
Time (hr)	^{3}H (% dose)	^{14}C	Time (hr)	^{3}H (% dose)	^{14}C
			0–2**	25.8	20.2
0–6*	33	19	2–4	5.7	<0.05
			4–6	4.2	†
			6–12	12.5	†
6–24	10.6	†	12–24	13.6	†
24–48	12.3	†	24–48	12.5	†
48–72	2.2	†	48–72	5.4	†
72–96	1.4	†	72–96	1.7	†
Total	60	19	Total	81	20

* Urinary $^{3}H/^{14}C$ ratio = 10.3.
** Urinary $^{3}H/^{14}C$ ratio = 7.2.
† No ^{14}C detected.

radioactivity was still in the form of glucoside. This was confirmed by almost complete hydrolysis of the material by almond emulsin and 90% inhibition of this hydrolysis by gluconolactone. The benzene extractable material obtained on hydrolysis contained ^{3}H but no ^{14}C. Thin layer chromatography indicated that 11% of this material was estrone, and the remainder was 17β-estradiol.

Peak 2 from the original DEAE-Sephadex column (0–0.8M NaCl) yielded 90% of the ^{3}H in an ether-soluble form after a 72 hour incubation with Ketodase. The hydrolysis was inhibited 95% by 7×10^{-4}M saccharolactone. Analysis of the fraction released by β-glucuronidase showed that 77% of the ^{3}H consisted of estrone and 17β-estradiol, while the remainder was composed of estriol and related triols, 2-methoxyestrone and material with the chromatographic mobility of the phenolic steroid ring D α-ketols.

DEAE-Sephadex chromatography (0–0.8M, followed by 0–0.4M NaCl) of the 6–24 and 24–48 hr urines gave evidence of partially separated peaks of ^{3}H behaving like phenolic steroid glucosiduronates. No glucoside-like material was detected beyond 0–6 hr. Smaller amounts of ^{3}H were detected in the steroid sulfate fraction. The glucosiduronate fraction over 6–24 hr was 81% hydrolyzed by Ketodase (95% inhibited by saccharolactone) and that over 24–48 hr was hydrolyzed only 62% by the β-glucuronidase preparation. These later glucosiduronate fractions showed increasing amounts of triols and other metabolites as compared with estrone and 17β-estradiol. Table 2 shows the pattern of steroids in the glucosiduronate fraction between 0 and 48 hours for subject MYL.

The 0–2 hr urine from BER, when chromatographed on XAD-2 resin, gave 5.39×10^{6} dpm ^{3}H and 0.696×10^{6} dpm ^{14}C ($^{3}H/^{14}C = 7.7$). DEAE-Sephadex chromatography (0–0.8M NaCl, 10 ml fractions) resulted in a number of peaks. Peak 1, fractions 4–9, contained 4.22×10^{6} dpm ^{3}H and 0.675×10^{6} dpm ^{14}C ($^{3}H/^{14}C = 6.2$); peak 2[4], fractions 15–20, contained 0.158×10^{6} dpm ^{3}H and 0.026×10^{6} dpm ^{14}C ($^{3}H/^{14}C = 6.1$); peaks 3–5, partially separated over fractions 22–31, contained 0.936×10^{6} dpm ^{3}H and no ^{14}C. These various peaks, together with a very small amount of ^{3}H in the steroid sulfate region (fractions 45–60), represented complete recovery of the radioactivity applied to the column.

Peak 1, corresponding to glucoside,

[4] The chromatographic peaks obtained for BER were much sharper than those for MYL, perhaps due to the smaller urine aliquots used in the former. This may have led to the apparent failure to detect peak 2 in MYL.

TABLE 2. Pattern of ^{3}H-metabolites (% total identified) in the urinary glucosiduronate fractions over 0 to 48 hours in MYL and BER

Steroid fraction	MYL	BER
Estrone	29	13
17β-Estradiol	11	12
Estriol	17	38
16-epiEstriol	8	7
Ring Dα-Ketols*	25	28
2-Methoxyestrone	10	2

* Probably contains some 2-OH metabolites.

showed, on further analysis, very similar results to those obtained with the corresponding material from MYL. The material behaved chromatographically as glucoside, was hydrolyzed by almond emulsin, and this hydrolysis was inhibited by gluconolactone. Between 10 and 15% of the aglycone released by hydrolysis was estrone, and the remainder was 17β-estradiol.

Peak 2 was intermediate in polarity between a monoglucoside and a monoglucosiduronate. Chromatography and enzyme hydrolytic experiments indicated that this material was neither a sulfate nor a glucosiduronate, and suggested that it was a diglycoside containing two non-acidic sugars. Because of the small amount of material, further characterization was not possible.

Peaks 3–5, pooled, yielded >90% hydrolysis with Ketodase (>90% inhibition with saccharolactone), and subsequent chromatography showed that 80% of the ^{3}H released was in the form of estrone and 17β-estradiol. Small amounts of the other various estrogen metabolites were also detected.

The 2–4 hr urine from BER yielded a pattern of radioactivity on DEAE-Sephadex chromatography (0–0.8M NaCl) as shown in Fig. 1. A small residue corresponding to glucoside was followed by major amounts of ^{3}H in the glucosiduronate region, followed in turn by smaller amounts in the sulfate region. The glucosiduronate peak was combined with suitable aliquots of urine collected between 4 and 48 hours

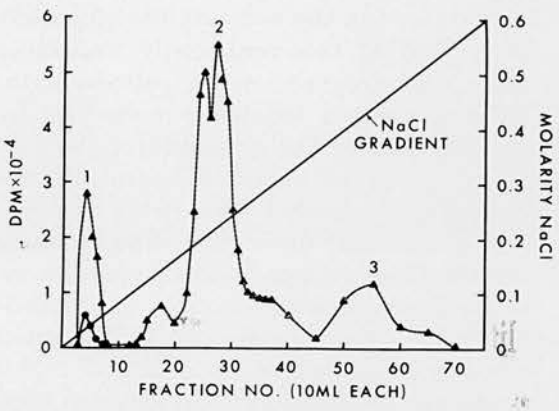

FIG. 1. DEAE-Sephadex chromatography of metabolites in 2–4 hr, subject BER. Peak 1 = glucoside, peak 2 = glucosiduronate, peak 3 = sulfate. ^{3}H = ▲---▲, ^{14}C = ●—●.

and incubated with Ketodase, resulting in 77% hydrolysis. The ^{3}H released was combined with that from the hydrolyzed glucosiduronate fraction from 0–2 hr and the whole was analyzed to yield the steroid pattern over the period 0–48 hr (Table 2).

In subjects MYL and BER, respectively, 46 and 41% of the ^{3}H in the urinary nonglucoside fractions (uncorrected for procedural losses) were found in the form of the various estrogen metabolites by the methods used.

Discussion

Some 75% of intravenously administered 17β-estradiol-3-glucosiduronate is excreted rapidly without deconjugation in the urine of the human female (4). A portion of this, however, undergoes direct dehydrogenation to estrone-3-glucosiduronate. A further 10 to 15% of the dose is excreted almost exclusively in the form of the 3-glucosiduronates of estrone and 17β-estradiol but representing material which has been deconjugated and reconjugated during its stay in the body (4).

In contrast to the metabolism of 17β-estradiol-3-glucosiduronate, it is apparent from the present study that 17β-estradiol-3-glucoside, following intravenous injection, is subjected to considerable deconjugation (hydrolysis) in that only 20% can

be recovered in the urine as the glucoside. Some 11% of this apparently undergoes direct dehydrogenation to estrone-3-glucoside as judged following hydrolysis by almond emulsin. The remainder of the dose appears to be subjected to hydrolysis, the released 17β-estradiol then being metabolized in a similar manner to intravenously injected 17β-estradiol. Evidence for this includes the increasing amounts of metabolites other than estrone and 17β-estradiol with time; the decreasing ability of β-glucuronidase to hydrolyze the excreted conjugates with time; the relatively small amount of urinary radioactivity identified as compared with the case of metabolites of injected estrogen glucosiduronates (4, 12, 13); and the final over-all pattern of urinary metabolites in the form of glucosiduronates which resemble those excreted following administration of 17β-estradiol (8).

In the rabbit, steroid glucosidase activity with a high specificity for the phenolic 3-glucoside has been found (14) and this activity is distinct from β-glucuronidase. It seems likely that a similar glucosidase or glucosidases may exist in the human, and the different pattern of metabolism of 17β-estradiol-3-glucosiduronate and 17β-estradiol-3-glucoside suggests that the glucosidase must act on the steroid conjugate either in a different organ or at a different subcellular site than does the glucuronidase.

It is of interest to note that intravenously administered 17β-estradiol-17-glucosiduronate in the human is deconjugated to the extent of about 65% as judged by urinary metabolites (Musey & Hobkirk, unpublished observations). However, unlike the case of 17β-estradiol-3-glucoside, there is no widespread metabolism of released 17β-estradiol as judged by the very small amounts of urinary metabolites other than the conjugates of estrone and 17β-estradiol following injection of the 17-glucosiduronate (12, 13).

Acknowledgment

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Metabolism of Intravenously Administered 17β-Estradiol-6,7-^{3}H-17-Glucoside in Normal Women

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Metabolism of Intravenously Administered 17β-Estradiol-6,7-^{3}H-17-Glucoside in Normal Women

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ABSTRACT. 17β-Estradiol-6,7-^{3}H-17-glucoside (E_2-17-glucoside) was injected iv into two normal young women. Within 72 hr, 61 and 64% of the ^{3}H dose was excreted in the urine in the two experiments. Within 2 hr of injection 0.7 and 1.9% of the dose was excreted as the unchanged glucoside together with 2.5 and 4.4% in the form of a double conjugate, identified with some certainty as E_2-3-glucosiduronate-17-glucoside. Between 2 and 4 hr after injection in one subject, and between 2 and 6 hr in the other, a lag in excretion of the label occurred, the small amount of ^{3}H found at these times being in all probability the above double conjugate. No unchanged monoglucoside was detected beyond 2 hr after injection and no double conjugate was detected with certainty beyond 6 hr. After 4 hr in one subject, and 6 hr in the other, the excretion of label increased markedly. Over the period 6–48 hr in each experiment only monoglucosiduronates were excreted and of these, 74% and 91% were composed of the 3-glucosiduronates of E_2 and of estrone (E_1) in the two studies. Smaller amounts of estriol, 2-methoxyestrone, 16-epiestriol and ring D α-ketol glucosiduronates were also present. It is suggested that E_2-3-glucosiduronate-17-glucoside, once formed, might be excreted in the bile with subsequent hydrolysis, and reconjugation largely in the form of the 3-glucosiduronates of E_2 and E_1. (*J Clin Endocr* **34:** 690, 1972)

RECENT work in our laboratory has led to the elucidation of the metabolism of iv administered E_2-3-glucosiduronate[3] (1) and E_2-17-glucosiduronate (2–4) in the human female. Further studies have involved the metabolism of E_2-3-glucoside in the human (5). This was done because glucosides of phenolic steroids have now been identified in both the rabbit (6,7) and the human (8) and it was of interest to compare the handling of the 3-glucoside in the human with that of the 3-glucosiduronate. Since there are now known to be considerable differences in the metabolism of these three conjugates of E_2 (3-glucosiduronate, 17-glucosiduronate, 3-glucoside) by the human (1–5), it was considered worthwhile to compare the picture obtained for the metabolism of iv injected E_2-6,7-^{3}H-17-glucoside. The study reported here concerns such an investigation.

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[3] The following trivial names for steroids and their conjugates are employed in the text: 17β-estradiol = E_2; estrone = E_1;
E_2-3-glucoside = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranoside;
E_2-17-glucoside = 3-hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranoside;
E_2-3-glucosiduronate = 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate;
E_2-17-glucosiduronate = 3-hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate;
E_1-3-glucosiduronate = 17-oxoestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronate;
E_2-3-glucosiduronate-17-glucoside = estra-1,3,5(10)-trien-17β-yl-β-D-glycopyranoside-3-yl-β-D-glucopyranosiduronate;
E_2-3-sulfate-17-glucosiduronate = estra-1,3,5(10)-trien-17β-yl-β-D-glucopyranosiduronate-3-yl-sulfate;
16-epiestriol = estra-1,3,5(10)-triene-3,16β,17β-triol;
2-methoxyestrone = 2-methoxy-3-hydroxyestra-1,3,5(10)-trien-17-one;
ring D α-ketols = 3,16β-dihydroxyestra-1,3,5(10)-trien-17-one + 3,17β-dihydroxyestra-1,3,5(10)-trien-16-one + 3,16β-dihydroxyestra-1,3,5(10)-trien-17-one.

Materials and Methods

E_2-17-β-D-glucoside. This compound was synthesized by a Koenigs-Knorr reaction as previously described for the preparation of 17α-estradiol-17-glucoside (6). The product had the

following properties: mp 175–185; $[\alpha]_D^{25} = 0°$; (C = 0.1, methanol), ν_{max}^{KBr} 2800-3500 (OH), 1500 cm^{-1} (aromatic); nuclear magnetic resonance (DMSO) 6.97 (H-1, 1 proton, d, $J_{1,2} = 8H_z$), 6.48 (H-2, 4, 2 protons, m, spacing $12H_z$), 4.91-4.15 (five sugar protons 1′, 2′, 3′, 4′, 5′). Analysis: Calc. for $C_{24}H_{34}O_7 \cdot \frac{1}{2} H_2O$ (443.54) C = 65.00, H = 7.96%. Found C = 65.17, H = 7.98%.

E_2-6,7-^{3}H-17-glucoside. This compound, of specific activity (SA) = 30 Ci/mmole, was prepared by reduction of the methyl ester of E_2-6,7-^{3}H-17-glucosiduronate (New England Nuclear Corp., Boston, Mass.) with $NaBH_4$. The method used was that of Collins *et al.* (7) except that the free phenolic group of the estrogen was blocked by acetylation prior to methylation by diazomethane in anhydrous benzene. Acetate groups were removed (6) and the product was purified by thin layer chromatography (TLC) on Silica Gel H in chloroform-ethanol (4:1). Constant SA was obtained after one crystallization with pure E_2-17-glucoside.

E_2-4-^{14}C-3-glucosiduronate. This was prepared by reducing biosynthesized E_1-4-^{14}C-3-glucosiduronate with $NaBH_4$ (9). All steps, including purification, have been published (1,5,9).

E_2-4-^{14}C-17-glucosiduronate. This conjugate was chemically synthesized by New England Nuclear Corp.

Reagents, etc. All organic solvents, materials for chromatography, enzymes, inhibitors and steroids were obtained, purified where necessary, and used as described elsewhere (9–11).

Injection of E_2-6,7-^{3}H-17-glucoside. The labelled material was first passed through Swinnex-13 filters of pore size 22 μ (Millipore Corp., Bedford, Mass.). Injection was performed in 10 ml of 10% (v/v) ethanol-saline (10) into an arm vein of each of 2 normal females at about day 10 of the cycle. Subject MCG (30 yr) received 25.5×10^6 dpm ^{3}H and subject PET (21 yr) received 19×10^6 dpm. Urine was collected in each case at 2, 4, 6, 24, 48 and 72 hr after injection and was frozen (—15 C) until required for analysis.

Analysis of urinary metabolites. Aliquots of urine (up to 6 hr) were processed on Amberlite XAD-2 resin (12) and then chromatographed on DEAE-Sephadex (A-25) in a linear gradient (0–0.8M) of NaCl (13). Peaks corresponding to E_2-17-glucoside were rechromatographed on A-25 in H_2O (13) and aliquots were extracted with ethyl acetate, then incubated with emulsin. Free E_2 was extracted with benzene.

Peaks from the salt gradient, behaving like E_2-3-glucosiduronate-17-glucoside, were incubated with Ketodase (β-glucuronidase), with and without saccharolactone, and the 17-glucoside formed was extracted with ethyl acetate after an initial benzene extraction. The presumptive monoglucoside was then chromatographed on A-25 in H_2O and aliquots were incubated with emulsin prior to benzene extraction. Further aliquots were methylated with dimethyl sulfate in borate buffer (14) prior to acid hydrolysis and identification of the E_2-3-methyl ether formed (3). Attempts were also made to remove the 17-glucoside moiety from the presumptive double conjugate by incubation with emulsin + saccharolactone (11), Mylase P (11) and a purified rabbit liver β-glucosidase preparation (15). The product was chromatographed on A-25 (0-0.4M NaCl) along with pure E_2-4-^{14}C-3-glucosiduronate.

Peaks from the original 0-0.8M NaCl gradient, behaving like monoglucosiduronates, were subjected to Celite column partition chromatography (CPC) in iso-octane:t-butanol:M NH_4OH (2:5:5) followed by the same solvents in the ratio of 2:10:10 (16). The eluted ^{3}H was mixed with pure E_2-4-^{14}C-3-glucosiduronate and E_2-4-^{14}C-17-glucosiduronate and rechromatographed on A-25 in a 0-0.4M NaCl gradient (13). The separated peaks were incubated with Ketodase, with and without saccharolactone, and the benzene-, or ether-soluble ^{3}H released was submitted to a Girard separation followed by CPC of the labelled aglycones (10).

All hydrolyzed steroids and their derivatives were crystallized to constant SA with appropriate unlabelled carriers (10). E_2-17-glucoside was crystallized in the conjugated form.

All radioactive counting was performed as previously described (10).

Results

Table 1 shows the excretion of ^{3}H in the urine of the two subjects. After excretion of about 5% of the dose in 2 hr, there was

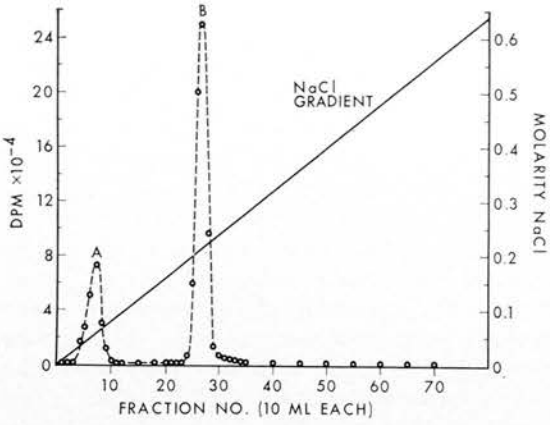

FIG. 1. DEAE-Sephadex (0–0.8M) chromatographic pattern of 0–2 hr urine after iv injection of E_2-6,7-^{3}H-17-glucoside into subject MCG. Peak A = E_2-17-glucoside; peak B = E_2-3-glucosiduronate-17-glucoside.

a lag phase (ca. 2–4 hr in MCG and 2–6 hr in PET) followed by an increased excretion of ^{3}H up to 48 or 72 hr.

In general the two experiments gave similar results. The ^{3}H in 0–2 hr yielded the chromatographic pattern shown in Fig. 1 (subject MCG). Peak A was identified as E_2-17-glucoside. Rechromatography of this on A-25 in H_2O gave a single peak between fractions 9 and 13 (5 ml fractions collected). (Pure E_2-3-glucoside appears between fractions 4 and 6). The presumptive 17-glucoside was extractable by ethyl acetate but not by benzene, and on TLC in chloroform-ethanol (4:1) had an R_f identical to authentic E_2-17-glucoside. It was 100% hydrolyzed (inhibited by gluconolactone) by emulsin to a benzene-soluble form identified as E_2 by TLC in benzene:ethyl acetate (7:3).

TABLE 1. Excretion of ^{3}H (% dose) in the urine following injection of E_2-6,7-^{3}H-17-glucoside into MCG and PET

Time (hr)	MCG	PET
0–2	4.7	5.7
2–4	1.2	0.93
4–6	3.8	1.2
6–24	23.6	26.4
24–48	18.8	22.7
48–72	12.2	3.8
Total	64	61

Ketodase incubation of Peak B rendered 3–6% of the ^{3}H extractable by benzene and 89–94% by ethyl acetate. In the presence of saccharolactone no ^{3}H was extracted by benzene and no more than 2% by ethyl acetate. The ethyl acetate-soluble ^{3}H from the uninhibited hydrolysis was eluted from A-25 by H_2O in a position similar to E_2-17-glucoside. An aliquot was 95% hydrolyzed by emulsin to E_2 (identified by crystallization). Another aliquot was crystallized with pure E_2-17-glucoside whereupon constant SA was obtained. A further aliquot, following methylation and hydrolysis, yielded E_2-3-methyl ether (identified by crystallization). None of the enzyme preparations used removed more than 2% of the glucose from C-17 of the intact double conjugate. After each attempted hydrolysis the ^{3}H-labelled product was eluted from A-25 in 0–0.4M NaCl over fractions (5 ml each) 64–74 whereas simultaneously chromatographed E_2-4-^{14}C-3-glucosiduronate appeared over fractions 73–81. On CPC in the 2:5:5 system the ^{3}H was eluted between holdback volumes (HBV's) 12–15 while the ^{14}C appeared at 8–10. In 0–2 hr 1.9% of the dose was excreted as unchanged glucoside in PET and the corresponding value for MCG was 0.7%. At the same time about 4.4% and 2.5% of the dose appeared as the double conjugate in the respective subjects.

In 2–4 hr for MCG and 2–6 hr for PET the ^{3}H excreted behaved like the double conjugate on A-25. In neither experiment was any E_2-17-glucoside detected at this time.

Over 4–48 hr for MCG and 6–48 hr for PET the A-25 pattern (0–0.8M NaCl gradient) exhibited ^{3}H solely in the region typical of phenolic steroid monoglucosiduronate(s). After CPC in the 2:5:5 system (ca. 96% of the ^{3}H eluted with this solvent; some 4% with the 2:10:10 system and methanol stripping), followed by A-25 chromatography in 0–0.4M NaCl with added E_2-4-^{14}C-3-gluco-

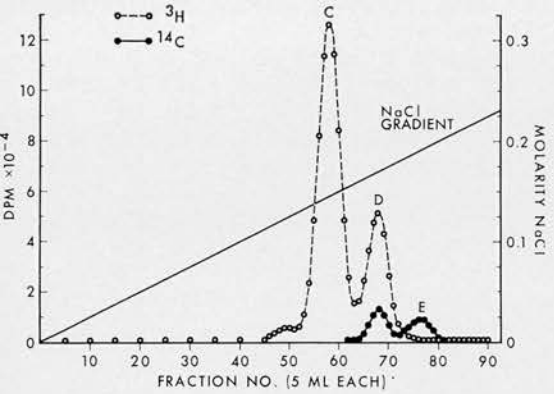

FIG. 2. DEAE-Sephadex (0–0.4M) chromatographic pattern of 6–48 hr urine after iv injection of E_2-6,7-^{3}H-17-glucoside into subject PET. Peak C = mainly ^{3}H-labelled E_1-3-glucosiduronate; peak D = mainly ^{3}H-labelled E_2-3-glucosiduronate (plus standard ^{14}C-E_2-3-glucosiduronate); peak E = standard ^{14}C-E_2-17-glucosiduronate.

siduronate and E_2-^{14}C-17-glucosiduronate, only two ^{3}H-labelled peaks were seen (Fig. 2 subject PET). Peak C was of a similar mobility to E_1-3-glucosiduronate while peak D was in the position of E_2-3-glucosiduronate. No ^{3}H coincided with standard E_2-4-^{14}C-17-glucosiduronate (peak E). Peak C was hydrolyzed 97–100% and peak D 89–94% by Ketodase (both inhibited 97% by saccharolactone). Of the ^{3}H released from peaks C and D by Ketodase in the 2 experiments, 83–88% was recovered after Girard separation and CPC. Peak C was 83–91% E_1 and peak D was 52–72% E_2. Table 2 shows the composition of the ^{3}H in the combined monoglucosiduronate fractions in 6–48 hr after crystallization to constant SA. Based upon sequential hydrolysis and extraction the maximum amount of double conjugate excreted in 6–48 hr was 3–4% of that in 0–2 hr; *i.e.*, ca. 0.1% of the dose.

Discussion

The metabolism of iv administered E_2-17-glucoside in the human female is clearly different from that of E_2-3-glucoside. Thus 20% of the latter is rapidly excreted unchanged in the urine and the remainder appears to undergo cleavage to E_2 which is metabolized similarly to iv injected E_2 (5). This is in accord with the finding that E_2-3-glucoside is an excellent *in vitro* substrate for rabbit liver glucosidase whereas E_2-17-glucoside is a poor one (15).

The metabolism of E_2-17-glucoside is similar in some respects to that of E_2-17-glucosiduronate although differing in others. Thus <2% of the former is excreted unchanged in the urine whereas 20–40% of the latter appears unchanged (4). The lag in ^{3}H excretion at about 2–6 hr after ^{3}H-E_2-17-glucoside injection is similar to that seen for ^{3}H-E_2-17-glucosiduronate (4). In the latter case this is due to biliary excretion of a sulfurylated form (E_2-3-sulfate-17-glucosiduronate) of the injected conjugate, together with another double conjugate (4; Musey and Hobkirk, in preparation), followed by slow intestinal reabsorption. In the case of injected E_2-17-glucoside the double conjugate formed is almost certainly E_2-3-glucosiduronate-17-glucoside which could conceivably be excreted via the bile with subsequent hydrolysis and reconjugation, before, during, or after reabsorption, with the formation of the 3-glucosiduronates of E_1 and E_2 as major metabolites. These latter 2 conjugates are the main urinary metabolites of biliary E_2-3-sulfate-17-glucosiduronate (4; Musey and Hobkirk, in preparation). The formation of small amounts of glucosiduronates of more highly oxygenated phenolic steroids suggests that some part of the original E_2-17-glucoside, or the double conjugate formed from it, might have been

TABLE 2. Pattern of ^{3}H-metabolites (% of total identified) in the urinary glucosiduronate fractions over 6–48 hr in MCG and PET

Steroid fraction	MCG	PET
Estrone	66	75
17β-Estradiol	8.1	16
Estriol	15.5	5
16 epiEstriol	2.6	1.5
Ring D α-Ketols	4.9	1
2-Methoxyestrone	2.9	1.3

hydrolyzed to E_2 which was then released into the main body pool.

It is interesting to note that the glucose residue at C-17 of the double conjugate found in this study was not removed by enzymes in the presence of the 3-glucosiduronate grouping, in spite of the facile removal of the latter group by Ketodase.

Acknowledgments

We are grateful to Dr. P. R. Blahey, Department of Obstetrics and Gynecology, The Montreal General Hospital, for his collaboration in this work.

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Metabolism of Intravenously Administered 17α-[6,7-^{3}H]Estradiol-17-Glucoside in Normal Women[1]

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WILLIAMSON, D. G., LAYNE, D. S., NILSEN, M., and HOBKIRK, R. Metabolism of intravenously administered 17α-[6,7-^{3}H]estradiol-17-glucoside in normal women. Can. J. Biochem. **50**, 958–962 (1972).

17α-[6,7-^{3}H]estradiol-17-glucoside was injected intravenously into two normal young women. Within 72 h, 60 and 46% of the ^{3}H dose was excreted in the urine in the two experiments. In the first 6 h, 4 and 1.4% of the dose was excreted as the unchanged glucoside, together with 14 and 5% in the form of a double conjugate, identified as 17α-estradiol-3-glucuronide-17-glucoside. Over the periods 6–24 h and 24–48 h none of the injected glucoside and only minor amounts of the double conjugate were present in the urine. The main excretory product after 6 h was 17 α-estradiol-17-glucuronide. This latter result contrasts with that obtained in previous experiments in which 17β-estradiol-6,7-^{3}H-17-glucoside was injected, and in which the main monoconjugates excreted were the 3-glucuronides of 17β-estradiol and estrone.

WILLIAMSON, D. G., LAYNE, D. S., NILSEN, M., et HOBKIRK, R. Metabolism of intravenously administered 17α-[6,7-^{3}H]estradiol-17-glucoside in normal women. Can. J. Biochem. **50**, 958–962 (1972).

Le 17α-[6,7-^{3}H]oestradiol-17-glucoside est injecté par voie intraveineuse à deux jeunes femmes normales. Après 72 h, respectivement 60 et 46% de l' ^{3}H injecté est excrété dans l'urine. Durant les 6 premières heures, 4 et 1.4% de la substance injectée est excrétée sans que le glucoside soit changé et 14 et 5% sous forme d'un double conjugué, le 17α-oestradiol-3-glucuronide-17-glucoside. Durant les périodes allant de 6 à 24 h et de 24 à 48 h après l'injection, rien du glucoside injecté et seulement de petites quantités du double conjugué sont présents dans l'urine. Le principal produit d'excrétion après 6 h est le 17α-oestradiol-17-glucuronide. Ce dernier résultat contraste avec celui obtenu dans des expériences antérieures où le 17β-oestradiol-6,7-^{3}H-17-glucoside a été injecté et où les principaux monoconjugués excrétés étaient les 3-glucuronides du 17β-oestradiol et de l'oestrone. [Traduit par le journal]

Introduction

In a recent study (1) we examined the metabolism of intravenously administered 17β-estradiol-17-glucoside[3] in the human female, and compared the results with those previously obtained when 17β-estradiol-17-glucuronide (2), 17β-estradiol-3-glucuronide (3), or 17β-estradiol-3-glucoside (4) were administered to similar subjects. Williamson and Layne (5) have shown that human liver and kidney tissue *in vitro* can form the 17-glucoside of 17α-estradiol, but not of 17β-estradiol, and it therefore became of interest to investigate the metabolism of 17α-estradiol-17-glucoside in the human.

Materials and Methods

Reagents and General Methods

All organic solvents and materials for chromatography were obtained, and purified where necessary, as described elsewhere (6, 7). Amberlite XAD-2 resin was purchased from Rohm and Haas, Philadelphia, Pa., and was washed extensively with methanol and water prior to use. Ketodase (beef liver β-glucuronidase) was purchased from Warner-Lambert, Morris Plains, N.J., and was used for incubation in concentrations of 125–500 U/ml in 0.1 *M* acetate buffer, pH 5, for 24 h at 38 °C. Saccharo-1,4-lactone (used as β-glucuronidase inhibitor at a concentration of 1 mg/ml) was purchased from Calbiochem, Los Angeles, Calif. Almond emulsin β-glucosidase (used for hydrolysis at a concentration of 0.2 mg/ml of 0.1 *M* acetate buffer, pH 5,

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[3]The following trivial names for steroids and their conjugates are employed in the text: 17α-estradiol-17-glucoside, 3-hydroxyestra-1,3,5(10)-trien-17α-yl-β-D-glucopyranoside; 17β-estradiol-17-glucoside, 3-hydroxyestra-1,3,5(10)-trien-17β-yl-β-D-glucopyranoside; 17β-estradiol-3-glucoside, 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranoside; 17α-estradiol-3-glucuronide, 17α-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronic acid; 17α-estradiol-17-glucuronide, 3-hydroxyestra-1,3,5(10)-trien-17α-yl-β-D-glucopyranosiduronic acid; 17β-estradiol-3-glucuronide, 17β-hydroxyestra-1,3,5(10)-trien-3-yl-β-D-glucopyranosiduronic acid; 17β-estradiol-3-glucuronide-17-glucoside, estra-1,3,5(10)-trien-17β-yl-β-D-glucopyranoside-3-yl-β-D-glucopyranosiduronic acid.

for 24 h at 38 °C) and glucono-1,5-lactone (inhibitor of glucosidase, used at a concentration of 10 mg/ml as described previously (8) were obtained from Sigma Chemical Co. Unlabelled steroids were purchased from Mann Research Laboratories, Inc., New York, N.Y.

All radioactive counting was performed, as previously described, on Nuclear Chicago liquid-scintillation spectrometers (6).

Preparation of 17α-[6,7-^{3}H]estradiol-17-glucoside

This material was prepared by the incubation of 17α-[6,7-^{3}H]estradiol with human kidney homogenate in the presence of UDP-glucose as described by Williamson and Layne (5). The material was extracted from the incubations with ethyl acetate and purified by thin-layer chromatography in chloroform–methanol (4:1). The specific activity was 21.7 Ci/mmol.

Injection of 17α-[6,7-^{3}H]estradiol-17-glucoside

The labelled material was first passed through Swinnex-13 filters of pore size 22 μ (Millipore Corp., Bedford, Mass.). No radioactivity was lost during this procedure. Injection was performed in 10 ml of 10% (v/v) ethanol–saline (6) into an arm vein of each of two normal females at about day 10 of the cycle. Subject AT (25 years) received 24.7×10^6 d.p.m. ^{3}H and subject KH (25 years) received 25.6×10^6 d.p.m. Urine was collected in each case at 6, 24, 48, and 72 h after injection and was frozen at −15 °C until required for analysis.

Analysis of Urinary Metabolites

Aliquots of urine were processed on Amberlite XAD-2 resin (9). An aliquot of 30 000 d.p.m. of 17α-[4-^{14}C]-estradiol-3-glucuronide prepared as described by Collins *et al.* (10) was added to each extract, and the extracts were then chromatographed on DEAE-Sephadex (A25) in a linear gradient (0–0.8 *M*) of NaCl (11). Material in peaks corresponding to 17α-estradiol-17-glucoside was submitted to ethyl acetate extraction followed by incubation with emulsin. Free 17α-estradiol was extracted with benzene.

The material in peaks from the salt gradient, with the chromatographic characteristics expected of 17α-estradiol-3-glucuronide-17-glucoside, was submitted to incubation with Ketodase, with and without saccharolactone, and the 17-glucoside so formed was extracted with ethyl acetate after an initial benzene extraction. Attempts were made to remove the 17-glucoside moiety from the presumptive double conjugate by incubation with emulsin and saccharolactone, Mylase P, and a purified rabbit liver β-glucosidase preparation (12).

The material in peaks from the 0–0.8 *M* NaCl gradient, with the chromatographic characteristics of monoglucuronides, was incubated with Ketodase, with and without saccharolactone, and the benzene-soluble tritiated material released was identified as 17α-estradiol by thin-layer chromatography (t.l.c.), and in some instances by recrystallization to constant specific activity with the authentic steroid.

Results

Table 1 shows the excretion of ^{3}H in the urine of the two subjects. In each case excretion was relatively rapid in the 0–6 h period and declined steadily thereafter (Table 1). The urinary recovery was 60% of the dose in subject AT and 46% in subject KH. The excretion was not necessarily complete at 72 h, when the collections were stopped in these experiments.

TABLE 1. Excretion of ^{3}H (percent dose) in the urine following injection of 17α-[6,7-^{3}H]estradiol-17-glucoside into subjects AT and KH

Time (h)	AT	KH
0–6	26.0	18.2
6–24	14.3	11.1
24–48	11.9	11.3
48–72	7.9	5.6
Total	60.1	46.2

The pattern of metabolites obtained in the urine from the two experiments was practically identical. The ^{3}H in the 0–6 h collections yielded the chromatographic pattern shown in Fig. 1 (subject AT), together with the ^{14}C pattern derived from the added sample of 17α-[4-^{14}C]-estradiol-3-glucuronide. Peak A was identified as 17α-estradiol-17-glucoside by chromatographic comparison with the authentic material (13). Emulsin caused 100% hydrolysis of the radioactive material to a benzene-extractable form, and this hydrolysis was inhibited by gluconolactone. The aglycone extracted into benzene after incubation with emulsin was identified as 17α-estradiol by t.l.c. in benzene–ethyl acetate (7:3). Peak A accounted for 4.0% of the dose in subject AT and 1.4% in subject KH.

The tritiated material in peak B comprised 14.0 and 5.1% of the dose in AT and KH, respectively, and was shown to be 17α-estradiol-3-glucuronide-17-glucoside. The identification of this compound was carried out as described by Hobkirk *et al.* (1) for the identification, in similar experiments, of 17β-estradiol-3-glucuronide-17-glucoside. Incubation of the material with Ketodase yielded 17α-estradiol-17-glucoside which was identified by recrystallization to constant specific activity with authentic material (13). The glucoside was in turn hydrolyzed by emulsin to yield an aglycone which was identical on t.l.c. with 17α-estradiol. The successive hydrolyses were inhibited by saccharolactone and by gluconolactone, respectively. None of

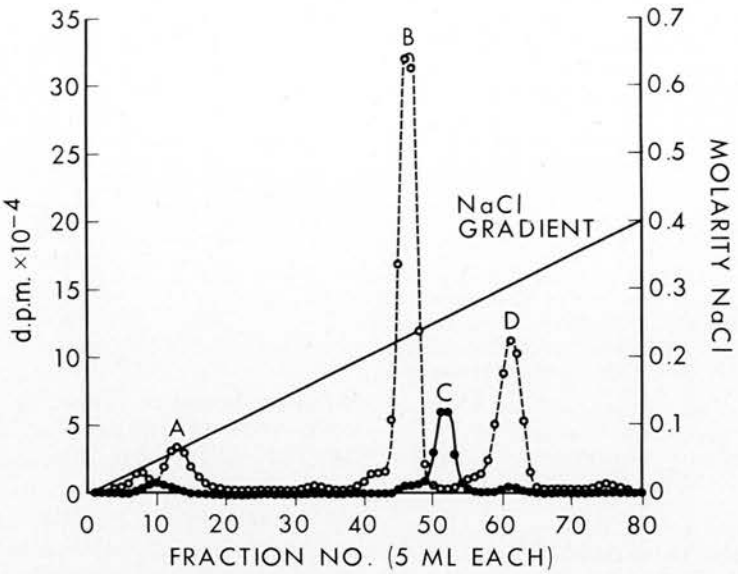

FIG. 1. DEAE-Sephadex chromatographic pattern of 0–6 h urine after intravenous injection of 17α-[6,7-^{3}H]estradiol-17-glucoside into subject AT. For identity of materials in peaks see text. (○) ^{3}H, (●) ^{14}C.

the enzyme preparations used was effective in removing the 17-glucoside from the double conjugate without prior removal of the 3-glucuronide.

Peak D (Fig. 1) consisted of a compound which was separated from 17α-[4-^{14}C]estradiol-3-glucuronide (peak C) but which migrated like a monoglucuronide on t.l.c. in chloroform–isopropanol–formic acid (5:3:1). The ^{3}H was extracted with benzene after Ketodase hydrolysis, and this hydrolysis was completely inhibited by saccharolactone. The aglycone consisted entirely of 17α-[6,7-^{3}H]estradiol as determined by t.l.c. and by recrystallization with nonlabelled material. Peak D contained 6.3% of the dose in subject AT and 3.4% in subject KH.

Continued elution of the Sephadex column beyond fraction 80 resulted in the recovery of a very small amount of tritium which probably represented double conjugates, such as a sulfoglucuronide, of 17α-estradiol.

Fig. 2 shows the chromatographic pattern of ^{3}H obtained when the metabolites in the 6–24 h urine collection (subject AT) were chromatographed on DEAE-Sephadex. Peak A (Fig. 1) was no longer evident, and peak B was extremely small. Peak D (17α-estradiol-17-glucuronide) now constituted the quantitatively major metabolite, amounting to 7.5% and 6.4% of the dose in subjects AT and KH, respectively. A small amount of a new tritium peak, C, had appeared. This material, which represented less than 2.0% of the dose, was coincident on the column with 17α-[4-^{14}C]estradiol-3-glucuronide, and also behaved as a monoglucuronide on t.l.c. The aglycone crystallized to constant specific activity with authentic 17α-estradiol.

The Sephadex chromatography pattern obtained with the 24–48 h urine samples was essentially similar to that in the 6–24 h collection (Fig. 2). The main metabolite was again peak D, with a small amount of peak C.

Discussion

The identification of the excreted conjugates, while not absolute, is reasonably firm. The material in peak A was identified as the compound injected, namely 17α-estradiol-17-glucoside, by chromatography and also by its hydrolysis with emulsin to yield an aglycone chromatographically identical with 17α-estradiol. The inhibition of this hydrolysis by gluconolactone is strong evidence for a β-D-glucoside, and the possibility that this might be the 3-glucoside of 17α-estradiol seems remote. These two glucosides of 17α-estradiol can, in fact, be separated on the t.l.c. system employed. In the case of the double conjugate in peak B (Figs. 1 and 2) the 17-monoconjugate moiety

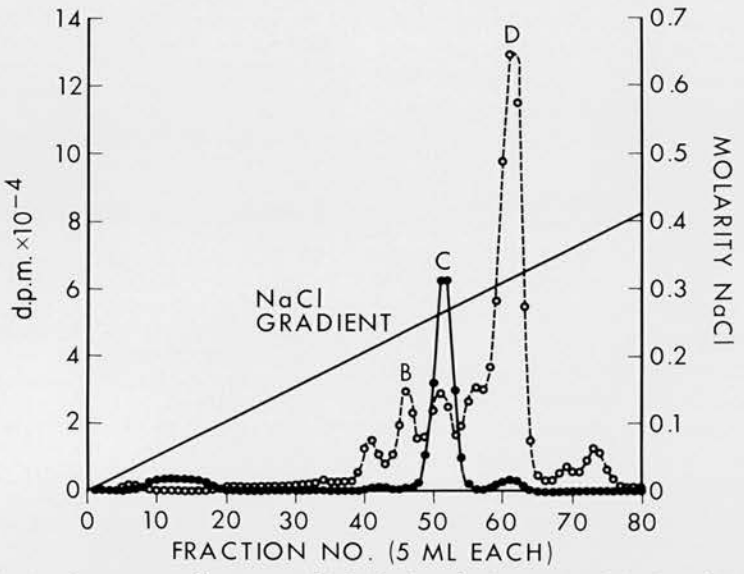

FIG. 2. DEAE-Sephadex chromatographic pattern of 6–24 h urine after intravenous injection of 17α-[6,7-^{3}H]estradiol-17-glucoside into subject AT. For identity of materials in peaks see text. (○) ^{3}H, (●) ^{14}C.

was firmly identified as 17α-estradiol-17-glucoside by crystallization to constant specific activity with the authentic compound. The removal of a conjugating group from the 3 position by Ketodase, and the inhibition of this hydrolysis by saccharolactone, is acceptable evidence that the 3 position of the steroid is conjugated with glucuronic acid.

The results indicate that 17α-estradiol-17-glucoside is partly converted to the 3-glucuronide and excreted as 17α-estradiol-3-glucuronide-17-glucoside (peak B, Fig. 1). A similar double conjugate with glucuronic acid at the 3 position is excreted in the early urinary collections after the injection of 17β-estradiol-17-glucoside (1). However, in the present work, *in vivo* removal of glucose from the 17 position led to the excretion of 17α-estradiol mainly as a monoglucuronide (peak D, Figs. 1 and 2), which appears to be 17α-estradiol-17-glucuronide on the basis of the following evidence. The material was separated on DEAE-Sephadex from authentic 17α-[4-^{14}C]estradiol-3-glucuronide (Figs. 1 and 2). Ketodase completely hydrolyzed the compound to yield 17α-estradiol, and this hydrolysis was inhibited by saccharo-1,4-lactone.

Several small peaks of radioactivity, which were not identified, can be seen in the chromatographic pattern shown in Fig. 2. It must be borne in mind that while these represent only small fractions of the injected dose, their relative quantitative significance is appreciable, and might well increase in the urine excreted after 72 h, which was not collected in this study.

The present results differ from those obtained after the injection of 17β-estradiol-17-glucoside, in that the monoglucuronide excreted in that case was 17β-estradiol-3-glucuronide (1). It appears likely that, in the human, the 17α-hydroxyl group is much more readily conjugated with glucuronic acid than is the 17β group. Sa'at and Slaunwhite (14) have demonstrated the *in vitro* formation of the 3-glucuronides of estrone and of 17β-estradiol by human liver homogenates. However, in unpublished work, we have been able to show the transfer, by human liver and kidney homogenates, of glucuronic acid from UDP-glucuronic acid to 17α-estradiol under conditions in which no formation of glucuronides of either estrone or 17β-estradiol was detected. It is possible that the 3-glucuronide of 17β-estradiol found by Hobkirk *et al.* (1), as well as the very minor amounts of 17α-estradiol-3-glucuronide which probably constitute peak C in the later urine collections in the present work, may be formed in the intestine following biliary excretion of liver metabolites. The 3-glucuronide could arise either by removal of glucose from the 3-

glucuronide-17-glucoside double conjugate, by complete deconjugation of the double conjugate followed by reconjugation with glucuronic acid at C-3, or by conjugation of free steroid formed by removal of glucose from the injected steroid-17-glucoside.

Williams and Layne (15) have reported that 17α-estradiol injected into human subjects is excreted largely unchanged, except for conjugation. A minor amount of metabolism in ring A takes place to form 2-methoxy-17α-estradiol, but the extensive ring D metabolism which converts estrone or 17β-estradiol to a variety of metabolites is absent. This is confirmed by the results of the present experiments, in that 17α-estradiol was the only steroid identified as an aglycone of the urinary conjugates investigated. It may well be that ready conjugation of the 17α-hydroxyl group by, for example, the liver, protects 17α-estradiol from further metabolism in ring D, and from dehydrogenase action which might convert it to estrone and 17β-estradiol. It would be of interest to know the site of this 17-conjugation, as to whether it occurs in the intestine from a biliary precursor or whether at an extra-enteric location. 17β-Estradiol-17-glucuronide is not, in contrast to the 3-glucuronide, an enteric metabolite of 17β-estradiol (16).

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